Proceedings of the Microbiological Research Group of the Hungarian Academy of Sciences

VOLUME I

Edited
by
GY. J. WEISZFEILER

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FOREWORD

DURING its activities over two years, the Microbiological Research Group of the Hungarian Academy of Sciences has attempted to fulfil its tasks as regards the study of micro-organisms and their effect on human and animal organisms, their immunological properties, and to help solve theoretical, medical and economic problems. These reports include our work on the problems of the variability of the tubercle bacterium, immunology and algae research which have not previously been published. In addition, the results and conclusions arrived at through discussions by research workers from the USSR, Czechoslovakia, Bulgaria and Hungary, taking place at our Institute, are included, concerning the attenuated *M. tuberculosis* vaccine strain No. 115 which was recommended by this Research Group.

Gy. J. Weiszfeiler
Director of the Microbiological
Research Group of the Hungarian
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CONTENTS

THE VARIABILITY OF THE TUBERCLE BACTERIUM AND THE PROBLEMS OF DARWINISM*

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DURING the last 20 years a profound change has ensued in the field of microbiology. The discovery of the role of deoxyribonucleic acid (DNA) as carrier of the genetic factors, of the helical structure of DNA, of the regulation of biosynthesis and, last but not least, the repressor system have laid the foundation of molecular biology. In all these successes microbiological research has played the greatest part. The greatest interest is focussed on bacterium genetics. Besides, investigation into the variability of micro-organisms may elucidate some problems of the historical development of the living matter. In this field Darwinism represents the fundamental science; it is the synthesis of the knowledge that mankind has collected in the fields of heredity and variability. However, in Darwins's time, i.e. about 100 years ago, microbiology as a science did not exist. Rensch's book "Evolution above the species level" (1959) as well as the numerous solemn sessions on the occasion of the centenary of the publication of Darwin's "The origin of species" (1959) left the results of microbiology out of consideration. Nevertheless, micro-organisms, owing to their rapid multiplication under well-defined conditions and their relatively simple structure, are especially suitable to elucidate profound rules of Darwinism. The parasitic property of micro-organisms represents not only a biological problem, but also an important medical one. Here we refer to the role of selection, the appearance of adaptation, the factors determining the dissemination of pathogenic micro-organisms, the stability of the properties, the origin and future development of these micro-organisms, and the ways of influencing them.

Research on the tubercle bacillus, by its correct name tubercle bacterium *(Mycobacterium tuberculosis)*, has produced numerous important results during the last few decades both in the field of biology and concerning variability. Though influencing its hereditary properties by DNA or in

* Read at the scientific Conference of the Austrian Association of Microbiologists and Hygienists and the Austrian Tuberculosis Association on 4 December, 1965.

developing successful new genetic models by bacteriophages or conjugation has not yet succeeded, the present knowledge on the variability of the tubercle bacterium has enabled us to develop definite opinions on numerous problems, and also to establish some rules.

I wish to attempt to elucidate in relation to the tubercle bacterium the variability of virulence, the resistance to chemotherapeutics, the phenomenon of dissociation, the non-acid-fast and the filterable forms and, finally, the very important problem of the atypical mycobacteria that have been isolated from both humans and animals.

The virulence of tubercle bacteria is their most important property which enables them to live under parasitic conditions. The virulence of a strain of micro-organism may be defined as follows: It is its capacity of multiplying and inducing pathological changes in a given animal species. Typing of tubercle bacteria is based on differences in their virulence for various animal species: the human type is virulent for man, the guinea-pig and the mouse; the bovine type is virulent, in addition, for the rabbit and cattle; the murine type is of weak virulence for man, for the guinea-pig and cattle, but it is virulent for the mouse. The avian type is virulent for the rabbit and for birds. Since virulence is a quantitative term, it must be determined as exactly as possible. At the beginning the most susceptible species, the guinea-pig, was only taken into consideration as an experimental animal. Recently, however, white mice and golden hamsters have also been used. Even a small dose of 0.00001 mg of a virulent strain produces generalized tuberculosis in guinea-pigs within 90—120 days. Strains not giving rise to progressive illness even when a dose as large as 10 mg is administered are considered to be of attenuated virulence. The vaccine strain BCG belongs to this group of strains.

As regards the epidemiology and clinics of tuberculosis, it is of great importance whether the severity, form and localization of the illness is determined by the virulence of the micro-organism, its affinity to certain organs, and its type. Numerous investigations carried out over 60 years by Steriopulo, Rolloff and Pagel, Opitz and Cheriff and many others as also in our laboratory have shown no relationship between virulence and clinical pattern. It could only be established that from chronic cases of skin tuberculosis, after many years of illness, tubercle bacteria of low virulence were often isolated. Similarly, Jensen has shown that from lupus cases, caused by the bovine type after several years of illness, transitional strains and, finally, the pure human type was cultured. Recently the observations of Mitchison et al. have called forth great interest. These authors isolated from patients treated with chemotherapeutics in South India strains

which proved to be of attenuated virulence for guinea-pigs. Correlation between severity of illness and virulence could not be established for these cases either. The change in virulence accompanying the acquirement of resistance to isoniazide should also be mentioned. Part of the resistant strains give rise to a pathological process in the guinea-pig which is progressive at the beginning, but shows regression subsequently. Hence, this phenomenon is also considered as attenuation.

The following questions have arisen: has the virulence of the tubercle bacterium declined during the 80 years while we have studied this microorganism? To what a degree is an attenuated or moderately virulent tubercle bacterium stable during animal passages? What is the antigenic structure of the attenuated strains in comparison with the virulent ones? Can a mutation of a single gene explain the changes in virulence?

The investigations carried out by numerous authors in Moscow and by ourselves (Weissfeiler and Morosova 1943) during 1930 and 1950 evidenced that the strains isolated from patients in that period were less virulent than those studied in the same town by Steriopulo at about 1900; 0.00001 mg of the latter being able to produce generalized tuberculosis in guinea-pigs even after 50—60 days. Thus, a significant decline of the virulence for the guinea-pig had ensued. The observations on the attenuated strains obtained in South India should be considered from the same point of view. It is of interest that we have isolated an attenuated strain from a monkey imported from India, suggesting that such strains are circulating there. From patients with lymphadenitis in the Yakutsk area we isolated four attenuated strains (Weissfeiler 1959). For the patients themselves these strains had not lost their virulence, suggesting that the parasitism of the strains was specialized for man to a still higher degree. This specialized parasitism is similar to that of the Vole Bacillus of Wells which is specialized for the vole, the strain being attenuated for man.

The second question, viz. the genetic stability of the attenuated virulence can be examined by submitting these strains to animal passages. We have tested 16 strains of tubercle bacterium that had been obtained in different experiments for the stability of their attenuated virulence. After 2 or 3 passages in guinea pigs or mice, four (25%) among the 16 strains became virulent while 12 retained their attenuated properties. One of the strains, viz. strain No. 115 which elicited good immunogenicity, was examined while it was carried over 7 consecutive passages in 40 guinea-pigs. Large doses up to 10 mg were injected into guinea-pigs intravenously (Fig. 1). The strain proved to be stable. Since these cultures were examined in monkeys as well and appeared to be more immunogenic than BCG, (Weissfeiler

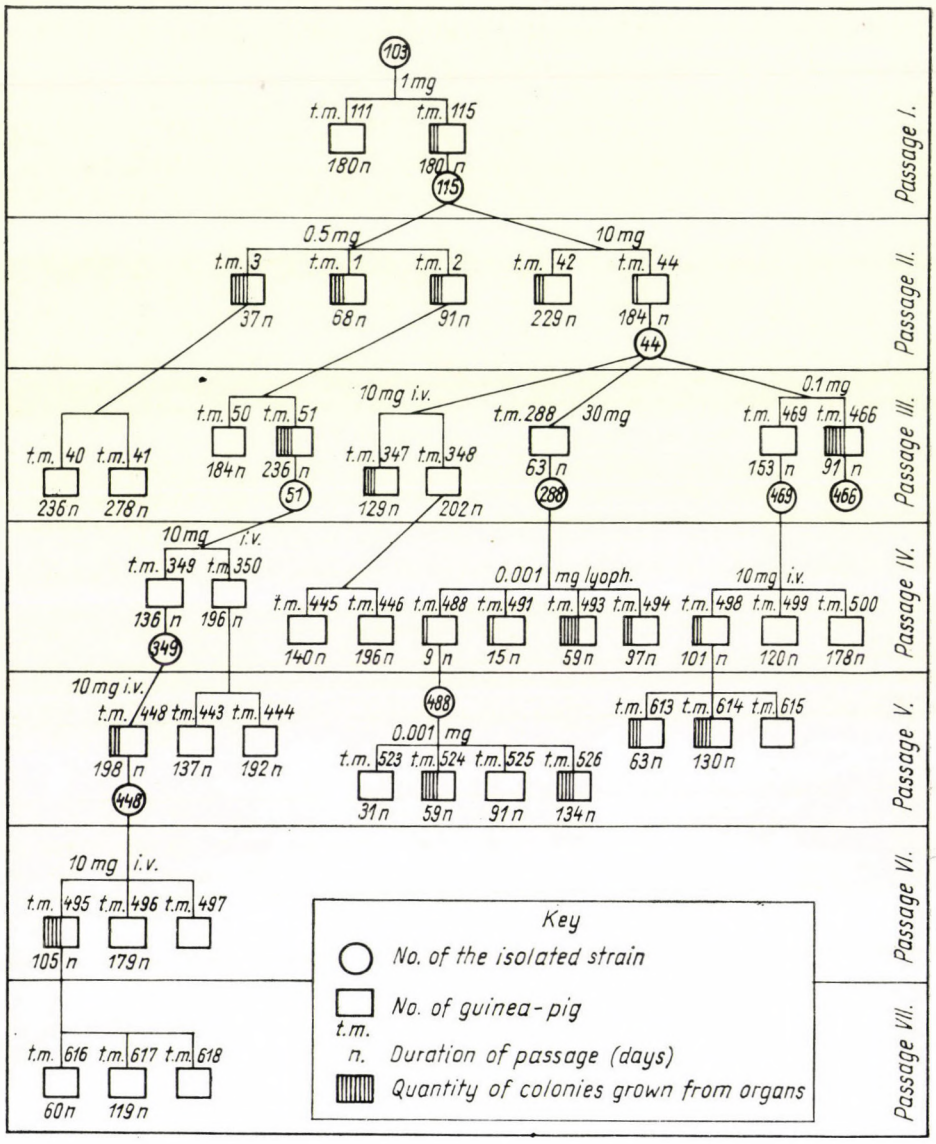

FIG. 1. Passage experiments on guinea-pigs for the study of the stability of the attenuated virulence of strain No. 115

1962) the strain is being studied thoroughly by several groups of investigators to decide whether it is suitable as vaccine strain. In another experiment the stability of a culture of moderate virulence was studied; no increase in virulence was observed in this case either. There are strains whose attenua-

tion is easily reversible. In contrast, others, like BCG and our strain 115, have lost their virulence irreversibly, the rule of mutation rate of 10^{-5} — 10^{-7} do not take place in this case.

The question of differences in the antigenic structure between virulent and attenuated strains has been studied by numerous investigators including us. Neither BCG nor strain No. 115 could be differentiated either by

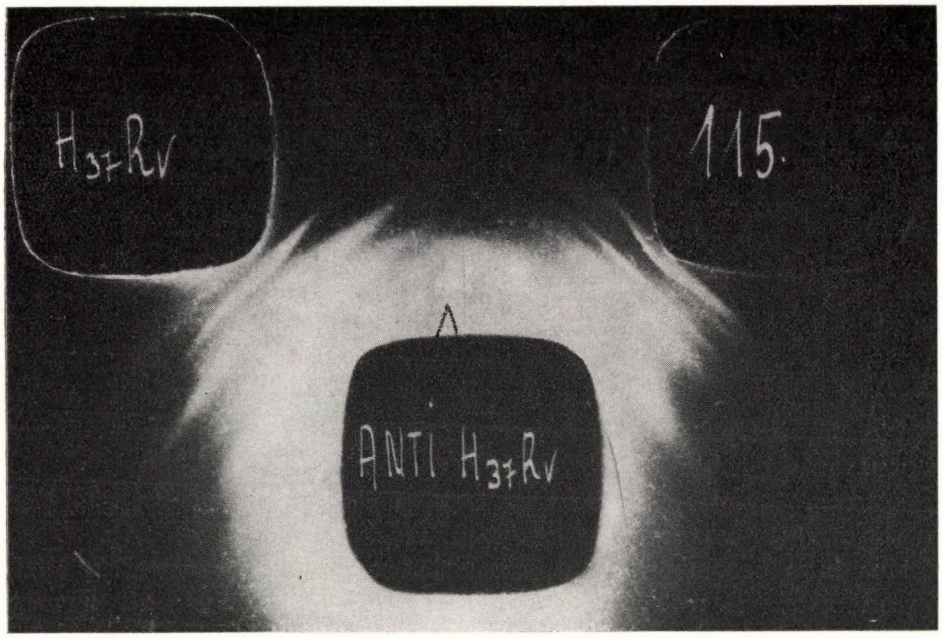

Fig. 2. Antigenic structure of strains BCG and No. 115 in gel-precipitation reaction

Ouchterlony's gel-precipitation method or by biochemical and cultural-properties (Fig. 2).

The loss of virulence can, therefore, must be considered to be a minus mutation which fails to influence the normal biochemical activity of the bacterium cells. This mutation is not reversible, at least not in the case of strains BCG and 115. For this reason we think that in these cases minus mutation of two or three genes has ensued. Phylogenetically it may be concluded that the human type has derived from the bovine one by loosing its virulence for cattle. The murine type might have arisen in a similar way. In the course of benign tuberculosis, e.g. skin tuberculosis or lymph-node tuberculosis, and historically in the course of pulmonal tuberculosis, too, the virulence may show a further significant decrease.

The second problem to be dealt with is the resistance of tubercle bacteria to chemotherapeutic drugs (drug resistance or chemoresistance). The term chemoresistance means the genetic ability of a bacterium to grow in an environment containing a substance of organic origin in a concentration which is bacteriostatic for the same bacterium species in general. Resistance is a quantitative, measurable property which plays a prominent role in modern bacterial genetics because it is transferable in transformation and conjugation experiments, and the frequency of its occurrence is easily controllable. It is an important property even from the medical point of view, because the cases caused by resistant bacteria cannot be successfully treated even with highly effective chemotherapeutics; these micro-organisms are able to spread to humans which is particulary serious problem as in the case of *Staphylococci* and *Shigellae*.

For the tubercle bacterium it was established soon after the discovery of streptomycin that resistance against very high concentrations (2000 mg/ml) can be developed (Youmans, Williston). Resistance of a high degree soon develops even to other chemotherapeutics, such as PAS and isoniazid (INH), both during therapy and during *in vitro* cultivation. Tubercle bacteria may become resistant to the new antituberculosis drugs, ethionamide, thiosemicarbazones, cycloserine etc. as well. Since it is generally accepted that drugs become ineffective against the strains that have developed resistance, attempts have been made to prevent resistance by combined therapy with two or more drugs administered simultaneously. When strains have become resistant, especially to the easily accessible INH, spreading of the resistant strains may lead to infections with primary resistant bacteria. Streptomycin resistance does not influence the virulence of the bacterium, whereas some of the INH-resistant strains show considerably attenuated virulence and several enzymatic changes, e.g. loss of catalase activity. The method of determination of resistance is of such great significance that the International Conference on Tuberculosis in Munich in 1965 discussed this question in detail; it is obvious that a uniform judgement should be based on uniform methods.

In the field of chemoresistance the writer and Karassova have examined the following questions:

1. What kinds of cytological changes can be observed by electron microscopy on the influence of INH and in resistant bacteria?

2. Is it possible to observe in the experimental animals the development of resistance due to the selective effect of INH?

3. What is the degree of stability of INH resistance in patients after therapy and after animal passages?

4. Is INH a mutagenic agent?
5. Is there any primary resistance in untreated patients?
6. What kind of virulence have the INH-resistant strains?

First a few words about the determination of resistance. We inoculated with sputum egg media containing 0.1—2.0 mcg/ml INH and control tubes simultaneously to determine the quantitative relationship between resistant and susceptible bacteria on the basis of the numbers of colonies grown on these media. This principle is at present generally applied. Special attention was paid on the time till the appearance of colonies on the media with and without INH. The colonies first observed to appear on the INH medium more than 10 days after growth on the control medium were considered not to derive from primary resistant bacteria, for theses produce colonies on the INH medium, as a rule, at the same time as or only a few days later then on the control one. The colonies with late appearance were regarded as originating from susceptible labile, easily adaptable cells, which underwent mutation after contact with INH in the medium. In this respect the strains obtained from different patients behaved differently. No primary resistance was observed in 20 patients. The strains showed even no apparition of partial resistance during the first 14 days of therapy. After this time resistance developed rapidly in the patients treated only with INH. After 90 days of therapy the bacteria proved to be resistant in 80% of the cases. At the same time only 11% of the bacteria were resistant in the patients given INH-PAS combined therapy. These findings have also evidenced that the development of resistance is hindered by combined therapy.

The stability of resistance was examined till the 581st day after termination of therapy. Though our material is not large, we have been able to show that resistance does not disappear and does not even decline during the first year. In several cases, surprisingly, the resistance increased. The resistance of the bacteria re-isolated from guinea-pigs after 86 days was the same as that of the inoculated culture. Thus, it is clear that neither the bacteria in the patients nor those in the guinea-pig passage represent a mixture of sensitive and resistant tubercle bacteria and that no back mutation into sensitive bacteria had ensued; if such bacteria had been present, these would have been demonstrable owing to their more active growth.

We examined in white mice whether any multiplication of resistant bacteria possibly present, as a result of selection after infection with INH-sensitive tubercle bacteria and subsequent treatment with optimal or suboptimal doses of INH occurs. The animals were infected with the strain BCG or No. 115 and treated with a daily dose of 0.2 or 2.0 mg of INH. After 15 and 30 days treatment, INH-containing and control media were

inoculated with samples from the lungs. Although the growth on the control medium was abundant, only few resistant colonies were obtained from the mice infected with strain 115 before treatment as well as after 15 and 30 days of treatment; they did not grow in number between the 15th and 30th day (Table I). BCG produced no resistant colonies. These results agree with

TABLE I

Growth of sensitiv and resistant colonies from mice treated with INH and controls

Inoculation	Doses of INH (days)	No. of animals	No. of colonies on the culture media		
			without INH	5 γ INH	20 γ INH
BCG	15	6	++++	—	—
	30	6	++++	—	—
	—	8	++++	—	—
115	15	6	++++	5	2
	30	6	++++	—	3
	—	8	++++	1	3

many other animal experiments in which resistance was not developed. As regards the experiments of Canetti and Grumbach, the growth of colonies up to the 53rd day were considered as manifestation of primary resistant cells. In our opinion this interpretation may cause confusion with the adaptation phenomenon that arises in the medium.

We have examined the virulence of our INH-resistant strains in guinea-pigs and white mice. Whilst the mouse-virulence of all the strains remained unchanged, eight of the 16 strains proved to be attenuated for the guinea-pigs. It was shown that these attenuated strains multiplied intensively till about the 45th day and even induced severe lesions which, however, regressed thereafter. We could not demonstrate correlation between the degree of resistance and that of the attenuation.

The lesions due to INH in the sensitive tubercle bacteria were examined by electron microscopy (Karassova). Bacteria were found to be swollen and filled with granular substance; subsequently these bacteria ruptured. In several cases granules freed from bacteria appeared to be multiplying and, perhaps, developing into resistant bacteria (Fig. 3). Granules reduced in size and cell walls about 7 fold in thickness were observed in INH-resistant bacteria.

Finally, the question is raised whether INH possesses only selective properties or also mutagenic ones. It is clear that the reduced virulence and

the loss of catalase activity are results of genetic changes not limited to the loss of INH sensitivity. In addition, we observed rapidly growing acid-fast variants in two cases. One of these variants appeared in the sputum of a patient during INH therapy, the second one in subcultures of a resistant strain in INH-containing medium. These strains when passaged proved to be

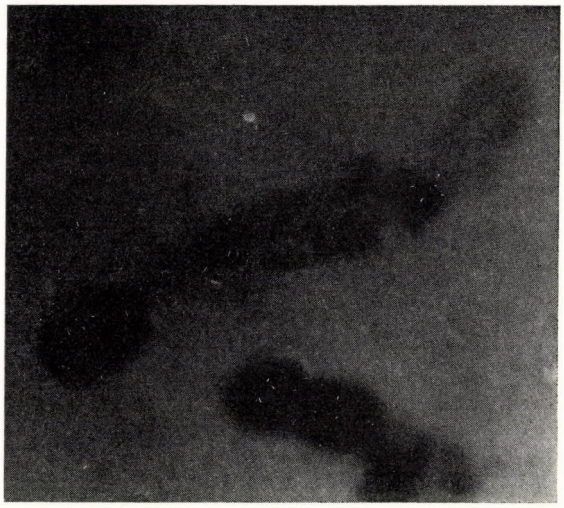

FIG. 3. Electron microscopy of tubercle bacilli damaged by INH and division of granula

unstable during the first generation, they produced tuberculosis in guinea-pigs and turned into typical tubercle bacteria.

Our observations allow some theoretical consideration. Appearance of resistance is undoubtedly a plus mutation. It is, however, questionable whether this is a spontaneous mutation which manifests itself owing to the selective effect of the chemotherapeutic drug. The fact that besides resistance other genetic markers, sometimes even a complex of these, change, proves that the bacteria are subjected at the same time to the mutagenic effect of the antibacterial substance. As a result of the mutagenic effect some cells that were first ihibited in growth will become resistant, i.e. begin to grow in the INH-containing medium. Adaptive genetic changes play a role here, especially in the case of labile strains. We have found that the degree of resistance increases progressively even after the therapy has been terminated, suggesting that the mutations which have been developed at the beginning in a defined direction lead to a further specialization according to new life conditions. The rare occurrence of back mutation in sensitive organism underlines the great danger which is represented by the spreading of drug-resistant tubercle bacteria.

The next problem concerning the variability of the tubercle bacterium is presented by dissociation. Petroff et al. have shown that like other bacteria tubercle bacterium is able to produce S colonies besides the typical, rough R colonies. The S variants, being different from the initial culture in several properties, remain stable over passages. Further, we demonstrated (Weissfeiler and Kalinina) in 1936 that chromogenic variants producing yellow pigment may dissociate from some cultures, and these variants also remain stable. It has been well known since long that some laboratory cultures that have grown on artificial medium for years may acquire saprophytic characteristics; they begin to grow faster and lose their virulence for experimental animals. In a strain we have observed the appearance of such a saprophytic variant twice; these variants grew abundantly even after 48 hours and even at room temperature. Another type of dissociation has been thoroughly studied by Jensen and Frimodt—Möller; variants resulting from dysgonically growing bovine strains suddenly began to grow abundantly, eugonically. In such cases the virulence of the eugonic variant does not change, but the metabolism of such strains certainly follows a new pathway.

Dissociation may ensue in man and in experimental animals as well. We observed such dissociation by culturing pigment-producing and S variants from organs of guinea-pigs which had been inoculated with cultures undoubtedly devoid of these variants. The writer and Morosowa published in 1943 the observation that pigment-producing colonies, besides typical ones of tubercle bacterium were grown from the lungs of a patient who died from tuberculosis. A pigment-producing variant was subcultured from these colonies. In another case a pure culture of S variant was obtained from the lungs. Vitschkanowa isolated a pigment-producing strain during streptomycin therapy from a case of tuberculous meningitis. Finally, the two cases in whom abundantly growing saprophytic strains arose under the influence of isoniazid should be mentioned again.

The variants resulting from dissociation are different from the initial cultures in numerous characteristics. They are not virulent for guinea-pigs and mice, they are resistant to streptomycin, INH and PAS and show some new properties, i.e. pigment-production, capacity of rapid growth and changed enzyme activity (Table II).

The newly acquired properties have proved to be genetically fixed, they remain unchanged over passages on media, and after animal passages the same strains can be recovered. Further dissociation resulting in new variants occurred only in few cases. Examination of the antigenic structure of the variants is of great importance, for in this way the numbers of retained and newly arisen components can be determined. These investigations, carried

TABLE II

Hereditary properties of the original and altered strains

Properties		H37Rv	M. avium	BCG—S	115—S	Ak.	Bov 8	918	230
R variants		+	—	—	—	+	+	—	—
S variants		—	+	+	+	—	—	+	+
Virulence	guinea-pigs	+	—	—	—	—	—	—	—
	mice	+	+	—	—	—	—	—	+
	rabbits	+	+	—	—	—	—	—	—
Resistance	INH	—	+ 50 γ	+ 50 γ	± 10 γ	—	—	+ 1 γ	± 10 γ
	Streptomycin	—	+ 50 γ	+ 50 γ	+ 50 γ	± 10 γ	—	+ 1 γ	+ 10 γ
	PAS	—	± 1 γ	+ 50 γ	+ 50 γ	+ 50 γ	—	+ 1 γ	+ 50 γ
Pigmentation		—	—	—	—	+	+	+	—
Amidase		3.5	5.6	5.6	5.6	—	3.5	—	—
Nitratereductase		+	—	+	—	+	—	—	+
Lipase		—	—	+	—	+		—	+
Arylsulphatase		+	—	—	—	+	+	+	—
Niacine		+	—	—		—		—	
Common antigens		10	6			6		6	
Immunogenicity		+	±	±	±			—	—

out by us first, demonstrated six antigens common in the pigment-producing variant Ak. and the H37Rv strain; in the latter strain 10 components could be demonstrated. Besides, examination of the immunogenic capacity against tuberculosis is of importance. Most of the variants examined by us for immunogenicity possessed a definite immunizing capacity. Although this capacity was less pronounced than that of BCG, it may be stated that the mycobacteria, unrelated to the tubercle bacterium, possess no immunizing capacity at all.

As regards the causes giving rise to variants, they do not arise as a result of mutation of one property, i.e. the rules governing the development of resistance cannot act here. We have demonstrated (Weissfeiler and Dwolaitskaia), that while a BCG suspension is stored in saline or ascites fluid, most of the bacteria lose their viability; after plating only few smooth or pigment-producing colonies grow out. From these colonies variants with new properties can be cultured. It is well known that ageing of the culture is a common factor of variant production. While studying the regeneration of filtrable forms (to be mentioned below), we obtained S variants from BCG and strain 115. At last, we have carried out a great number of experiments with tubercle bacteria which had practically lost their viability under the

influence of phenol, heating to 100° C or acetone. These bacteria which failed to grow on the usual media, were injected into newborn mice to give as favourable conditions as possible for growing, and so for the re-establishement of viability. From the animals haemolyzed blood medium and Löwenstein medium were inoculated and some cultures were again passaged in mice. In this manner cultures of S variants were obtained after 3—7 months. The suspicion that the mice had been spontaneously infected by acid-fast mycobacteria could be excluded, since from healthy mice no mycobacteria could be isolated and some of the S variants were immunogenic against tuberculosis. All these observations suggest that variants may occur as a result of autolysis, againg, production of filtrable forms or damaging of the bacteria by saline, acetone etc. Bacterial autolysis giving rise to variants may occur, in spite of active tuberculosis in man, too. This is probably due to the disappearance of some repressors which had inhibited the functions of certain enzymes being more resistant. Here some characteristics, lost long ago in the course of phylogenesis, may manifest themselves; these are identical or greatly similar to the characteristics of the avian tubercle bacteria in the case of S variants, and to saprophytic mycobacteria in the case of chromogenic and saprophytic mycobacteria.

As to the theoretical aspects, the rise of variants having numerous new properties, while having lost numerous others, should be considered as a special form of mutation which is characteristic of micro-organisms. Most mutants show an atavistic character which is reminiscent of phylogenetically older properties. We therefore believe, that it would be more correct to use the new term plurifactoral or ancestral mutation. Modern genetics do not deal with this phenomenon, probably because of its complexity. Nevertheless, it is of extraordinary importance today, even from the practical point of view, in connection with the problem of atypical mycobacteria. In this respect the question should be raised where these micro-organisms have come from. We believe that part of the atypical mycobacteria isolated from man represent variants of the tubercle bacterium. The great resemblance between the avian tubercle bacterium and the S variants suggests that the S variants represents a historically more primitive ancestor of the tubercle bacteria of mammals. The question is whether variants resulting from plurifactoral mutation show the direction of the future evolution of tubercle bacteria. We believe that the increased resistance to damaging influences including chemotherapeutics is favourable for some of the variants as regards further multiplication either in the human body or in animals or in the outside world. An arisal of a new pathogenic species immediately from these variants seems to be improbable.

The following problem, viz. the possibility of transmutation into non-acid-fast forms, is the most complicated question of the variability of the tubercle bacterium (Weissfeiler 1933). We associate this problem, in light of most recent research, with the question of the filtrable and L forms of this micro-organism. The question whether the tubercle bacterium undergoes pronounced genetic changes to such an extent that it loses the characteristics of the Mycobacterium family reminds us of the times of Nägeli's pleomorphism. Recently, however, development of L forms by various micro-organisms has been observed; the resulting L forms are similar to the mycoplasmas and differ from the original bacterium to such an extent that there exist neither serological nor biochemical reactions indicative of their origin. These observations were achieved first of all by Klieneberger—Nobel and by Dienes.

The question of non-acid-fast forms emerged sometimes as a sensational discovery which, however, could not be proved later. I would like to mention Ferran's alpha bacillus, a gram-negative bacterium with flagella, from which even vaccine against tuberculosis was produced. Another error originated from Enderlein, who stated the transformation into aspergillus fungi. At that time I visited Enderlein. It turned out that Enderlein was a botanist, who did not recognize the methods of pure bacterial cultures; nevertheless, he attributed to the tubercle bacterium beautiful pictures of cyclogenia of fungi seen under the microscope. These and other similar occurrences explain the scepticism concerning this question.

During our work we made efforts to avoid confusion with contaminants. We have demonstrated that under cultural conditions inadequate for the tubercle bacterium certain laboratory strains (called labile strains by me) growing like S variants multiply in non-acid-fast form; these will become acid-fast again when inoculated into adequate media. We attribute these changes to non-genetic modification. Starting from the idea that slow-growing non-acid-fast mutants are not recognized in the acid-fast population, we allowed a suspension to stand in water, and inoculated petri-dish media from the suspension. In some instances small colonies with coccoid or diphtheroid micro-organisms grew out. These micro-organisms which grew poorly in subcultures, might be considered to have been L forms. In another series of experiment with the culture of strain K6-a, rapid-growing Gram-positive rods were observed. If these rods had resulted from latent contamination they would have been recognizable, owing to their rapid growing, even in the first generation. We examined strain K6-a for immunizing capacity against tuberculosis and the possibility of its reversion into acid-fast culture. The strain was not immunogenic,

but when it was injected into guinea-pigs, together with killed tubercle bacteria, acid-fast cultures were obtained from the animals. Accordingly, a reversion into the original mycobacterium had ensued. In my laboratory Vitchkanova obtained non-acid- fast cocci producing pale red pigment besides colonies of typical tubercle bacteria from the cerebrospinal fluid of a child with tuberculous meningitis. The cocci grew slowly and in the first four subcultures colonies of typical tubercle bacteria appeared. We made efforts to free its culture from latent tubercle bacteria by subculturing from single colonies. The cultures so obtained repeatedly produced tuberculosis in guinea-pigs. The cocci contained acid-fast granules. The strain classified as mycococcus which is a genus recently described by Krassilnikow; this genus is the simplest representative of the *Actinomycetaceae* family. I would like to mention Tarshis's work (1958). This author observed a yellow pigment-producing, non-acid-fast culture after consecutive subculturing of a streptomycin-resistant strain of the tubercle bacterium. Mattman et al. (1960) often obtained stable L forms from cultures of tubercle bacteria which consisted of coccoid and mycelia-producing elements. Basserman (1955), examining under the electron microscope an originally virulent, but in subcultures pleomorphic, non-acid-fast culture saw spherical L forms. All these facts, in addition to numerous earlier works (Arloing, Vaudremère, Mellon and others) favour the possibility of atavistic or ancestral mutation of the tubercle bacterium into phylogenetically surpassed forms.

It is well known that the L forms of micro-organisms contain small elementary bodies which pass through the bacterial filters. On this basis the question of the filtrable forms of the tubercle bacterium may be connected with that of the L forms. This subject, owing to the activities of Vaudremère, Calmette and Valtis, was in the centre of interest 30 years ago; since then, however, it went out of fashion. The following sentence in Topley and Wilson's manual is illustrative: "It would be unreasonable to discuss in detail observations which probably resulted from erroneous techniques." We do not agree with such an interpretation. It is hard to believe that Calmette, one of the most prominent microbiologists of the present century, employed the bacterial filtration technique erroneously. Besides, more recent works of the Calmette school, viz. those by Boquet and Negre (1956) have shown that the so-called incompletely developed forms of the tubercle bacterium pass through bacterial filters.

The sceptic or negative standpoint was due to the lack of an exact, adequate and easily controllable method for the demonstration of filtrable forms. For this reason I attempted to elvolve such a method. Starting from the assumption that the filtrable forms arise in the course of autolysis, I

made by gentle shaking surface cultures of BCG settle on the bottom of the fluid medium and placed the cultures in the incubator. After 48 hours the culture fluid was filtered and inoculated into media to control the absence of normal bacteria. To concentrate the filtrable forms, I adsorbed them from the fluid to aluminium hydroxide. The adsorbed filtrate was injected into guinea-pigs and mice to afford an opportunity for *in vivo* regeneration. In reproducible experiments we succeeded in culturing acid-fast strains from the animals. The strains so obtained proved to be different from the starting culture, some of them were S variants. Based on these results we have considered the existence of filtrable forms to be proven and accepted that from filtrable forms regeneration of bacteria with new characteristics may occur.

Further, we examined with our adsorption method whether filtrable forms are present in the blood of patients suffering from tuberculosis. The writer and Danilova found paraspecific lesions in the guinea-pigs inoculated with the adsorbed blood. The lesions were characteristic of the abortive tuberculous infection described by the French school. From one of these cases the vaccine strain No. 115 with attenuated virulence was obtained from the inoculated guinea-pig.

In our opinion the filtrable forms, which arise during autolysis or result from some other impairments, are particles of the tubercle bacterium, still capable of regeneration. The most favourable conditions for regeneration are present in the animal body after phagocytosis; in this respect the filtrable forms are similar to the viruses which find in the phagocytes all the building stones and enzymes necessary for their intracellular biosynthesis. The fact that the regeneration results in the apparition of individuals of changed properties suggests that the processes of the plurifactorial mutation or dissociation might be due to a similar mechanism.

Finally, the problem of atypical mycobacteria should be discussed. This is, in close relation with the problem of variability and it raises many questions which have been clarified to a certain extent during the decades while the tubercle bacterium has been investigated. It has often been evidenced with the perfection of the cultivation methods that from man, besides typical tubercle bacteria, mycobacteria with variable characteristics can be isolated. I should like to point to the important work of Löwenstein's school in Vienna which called, and deserved, great attention concerning tuberculous bacteraemia. Acid-fast mycobacteria were isolated, besides the tubercle bacterium from the blood, in fact not so frequently as Löwenstein had published, by Popper, Coronini, and Saenz, in the USSR by Berman and Gelberg. Some authors have concluded that these mycobacteria

are saprophytes; others, including me, classify them as variants of the tubercle bacterium. The observations of Pollak and Buhler brought a new turn in 1952. These authors reported on fatal cases from whom mycobacterium strains, so-called "yellow bacilli", were isolated. These strains produced no pigment in darkness, but their colonies became yellow after several hours of illumination. This kind of mycobacteria was, therefore, given the name

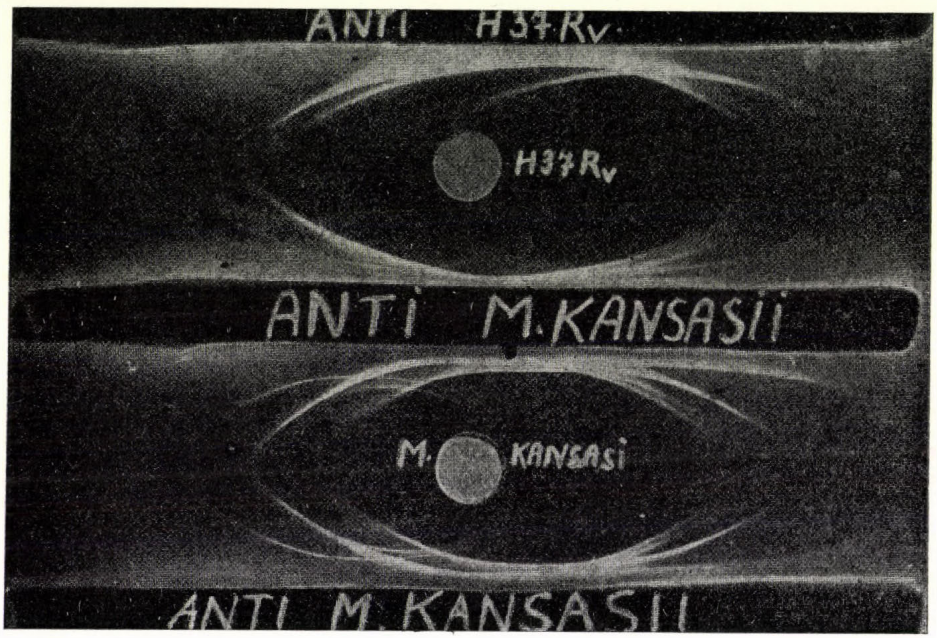

FIG. 4. Antigenic structure of *M. kansasii* (strain 232) by immune electrophoresis

"photochromogenic". Subsequently, numerous investigators isolated similar strains which were then separated as an independent bacterium species, *M. kansasii*. Without proposing any new order or classification for the atypical mycobacteria, Runyon recommended a grouping according to pigment production and growth rate; in addition to the group of photochromogenes, three groups, i.e. scotochromogenes, non-chromogenes and rapid growers were proposed. The questions whether these are mycobacteria, related to the tubercle bacterium or not, whether they are independent aetiological agents of hitherto unknown species, or bacteria accidentally multiplying in the body, could be the subject of a complete paper.

In the following the *Mycobacterium kansasii* is to be discussed. It is of weak virulence for the guinea-pig, but virulent for mice. It forms smooth S

colonies. Its immunizing capacity against tuberculosis, as shown by us, agrees with that of BCG which is a very good vaccine strain. By the gel-precipitation technique and immunoelectrophoresis we have demonstrated 6, Ouchterlony and Norlin demonstrated even 9, antigen components common with the tubercle bacterium (Fig. 4). We were the first to report (International Congress on Tuberculosis, 1963) that non-pigment-producing, white R variants could be obtained from this strain; this finding was confirmed by Runyon, Hauduroy and Tacquet. On the basis of its properties this mycobacterium may be considered as a new type of the tubercle bacterium which, having lost its virulence for the guinea-pig, represents a further specialization towards man and shows a well-defined chemoresistance to most of the antibacterial drugs. It is possible that *M. kansasii* arises under the influence of chemotherapy. Its close relation to the tubercle bacterium is doubtless.

Scotochromogenic mycobacteria present the greatest difficulties. We do not agree with Bönicke who believes that these are saprophytes *(M. aquae)*. Firstly, we have succeeded in obtaining pigment-producing variants even from *M. kansasii*. Moreover, out of the four strains that were kindly supplied by Hauduroy, two show, in contrast to the saprophytes, moderate but well-defined immunizing capacities against tuberculosis. The other two have no immunogenicity, but strain 918 has six antigens in common with the *M. tuberculosis* strain H37Rv. This finding also supports their close relationship. Finally, it should be pointed out that pigment-producing variants can be obtained both from typical tubercle bacteria and the avian type. The scotochromogenic mycobacteria, at least some of them, are variants of pathogenic strains. It may, nevertheless, occur that *M. aquae* invades the oral cavity and, perhaps, it may vegetate even in the cavern.

The third group of non-pigment-producing mycobacteria is called avian-like or Battey bacillus. These bacteria are S variants as shown by their cultural characteristics. Data in the literature (Freerksen etc.) suggest that these strains cannot be differentiated from the avian strains on the basis of their antigenic structure and, they possess a well-defined immunogenicity. Strikingly, avian-like strains have been obtained from dust and from the soil as well. These are either S variants of tubercle bacteria or facultative pathogenic mycobacteria widespread in Nature.

The fourth group of atypical mycobacteria, the rapid growers, probably include still more heterogeneous strains. These may be considered as saprophytic variants to originate from tubercle bacteria as well as from the above three groups of atypical mycobacteria. No doubt there are also true saprophytes among them. In our opinion systematization and classification of all the

atypical strains and the elucidation of their relation to the tubercle bacterium need a thorough antigen analysis. Several, hitherto unknown, species of mycobacteria must exist which may invade the animal organism and multiply there. The saprophytic *M. diernhoferi*, described by Bönicke, is a representative of these strains.

The problem of facultative pathogenic mycobacteria lies near to the laws of Darwinism in the micro biology, because the acquirement of pathogenicity by a saprophytic species is thought to follow the same way over facultative parasitism in which the tubercle bacterium acquired its pathogenic capacity. In this relation our observations on atypical mycobacteria occurring in monkeys may be of interest. Two accidental observations prompted us to investigate the occurrence of atypical mycobacteria in monkeys systematically. In one instance we examined a tubercle bacterium of low virulence, in the other a new mycobacterium. The latter, called strain Zol. proved to be completely different from the tubercle bacterium in its antigenic properties. Further studies with 69 *Macacus rhesus* and Cercopithecus monkeys imported from India and Abyssinia, (their kidneys were used for tissue culture production) led to unexpected results. From 33 animals 50 strains of mycobacteria were obtained. These strains were studied thoroughly. Of them 9 proved to be photochromogenic, 4 scotochromogenic, 13 non-pigment-producing, and 4 rapid growers. Most of the strains grew dysgonically, i.e. slowly at 37° C; after 15—30 days they consisted of very short, almost, coccoid acid-fast bacteria. The examination of their multiplication capacity in the mouse and of their mouse virulence gave very interesting results (Table III). All the strains except one multiplied in the animal, some of them caused severe lesions in the kidneys, lungs and, rarely, in the spleen. The monkeys were healthy clinically. We consider these micro-organisms to be facultative pathogens, able to cause illness under special circumstances even in their natural host, the monkey. Runyon's classification cannot be used even here; new variants with changed pigment production and rapid growth separated themselves during the short period of experiments. Here we have encountered a new species: *Mycobacterium simiae*. In addition, some of the strains probably belong to the avian group.

As a result of our investigations three directions of the hereditary variability of micro-organisms might be distinguished.

1. Minus mutation. Loss of virulence of the tubercle bacterium, appearance of auxotrophes leads to a specialization.

2. Plus mutation. Acquirement of chemoresistance which represents an adaptation to new life conditions; it enables the micro-organism to multiply in the presence of harmful substances.

Table III

Behaviour of the monkey strains in mice

Group	Strains	Cultures from mice		Pathological changes in organism of mice					Dead after inoculation (days)
		Days after inoculation	No. of colonies	Day	Lungs	Spleen	Kidney	Liver	
I	5	65	+++	65	—	—	—	—	—
	14	27	++++	75	++	—	—	—	—
	20	65	++++	65	++++	+	+	±	—
	25	90	+++	90	+++	—	—	—	—
	27	90	++++	90	+++	—	—	—	—
	29			34	±	++	++	—	9, 28, 34, 34, 34
	52	63	++++	60	++++	—	—	—	34, 34
	61	61	+++	84	±	+	—	—	—
	68	73	++++	73	+	+	±	—	—
II	32	65	++	65	±	—	—	—	—
	47	65	+++	61	±	+	—	—	22
	51	60	++++	60	+++	+	—	—	22, 34, 36
	64	52	++	52	—	—	—	—	6, 11, 20, 32, 40, 52
III	3	53	++	90	—	+++	—	++	11, 22, 51, 63
	23	63	+++	63	—	+	++++	—	—
	46	57	++++	70	—	+	—	—	9, 14, 19, 63, 67
	53	33	++++	46	—	++	±	—	—
	54	33	++	46	—	+	—	—	—
	59	50	++++	35	+++	+	+++	++++	10, 13, 19, 31, 34, 46
	63	70	++++	70	—	+	+++	—	4, 7, 8
IV	1	90	+	90	—	—	—	—	—
	4	90	++++	90	—	—	++++	—	22, 33, 39, 56, 90
	15	51	+++	53	—	—	++++	—	20, 20, 21, 13, 14, 18
	55	77	++++	77	—	—	—	—	—

3. Plurifactoral mutation or ancestral mutation. This term includes dissociation, appearance of pigment-producing, saprophytic, non-acid-fast forms and L forms. Significant changes ensue in a number of characteristics; some of these mean new properties, others loss of old ones. These changes usually lead to an increased viability of the bacterium in Nature. In this way a great number of new variants different from one another in some properties arise; the resulting variants afford good material for natural selection.

The rules of the historical evolution of the tubercle bacterium which have been clarified to some extent by our observation, might be summarized as follows. No doubt that the pathogenic mycobacteria have developed from saprophytic mycobacteria, some of which are still widespread in the soil. As intermediate step, there exist facultative pathogenic micro-organisms able to multiply in the animal body as well as in free nature. The avian tubercle bacterium has developed from such micro-organisms in birds which find their foods in the soil; carnivorous animals when eating infected birds were infected by these bacteria and so they have become adapted to mammals. First the bovine type arose which is pathogenic for all mammals. The human type represents a further step of evolution; it has lost its wide virulence range and become specialized for man. Over thousands of years the human tubercle bacterium found favourable conditions for its further spread. The peak of its evolution was reached in Europe 80 years ago and in the colonies several decades later. Owing to the great scientific achievements and sanitary measures, the conditions for the existence of the tubercle bacterium have significantly worsened since the beginning of the 20th century, especially after World War II. The discovery of new chemotherapeutics is of special importance, but the role of the rise in living standards of the population of some countries has also been significant. In numerous countries the bovine type has been remarkably suppressed or even eradicated. The domination of the tubercle bacterium has become limited but it still causes heavy losses in the population in Asia, South America and Africa. The danger of outbreak of new cases has not ceased even in the developed countries, owing to the adaptation to chemotherapeutics and to the appearance of new species like *M. kansasii*. The fight against these micro-organisms has not been decided. We must, besides using the already approved methods, set ourselves a new target, viz. hindering the spread of new adaptation forms.

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REACTIVATION IN NEW-BORN MICE OF MYCOBACTERIA INACTIVATED BY PHENOL, HEAT OR ACETONE

by

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VERY recently great interest has been aroused by the observation that in pathological material (sputum, lungs, pus) of patients suffering from tuberculosis acid-fast bacteria can be demonstrated which fail to grow *in vitro* and do not cause tuberculosis in the guinea-pig. Griffith (1924) failed to obtain any cultures from 97 out of 176 cases of lymphadenitis with microscopical evidence of acid-fast bacteria. Feldmann (1945) was unable to induce tuberculosis in guinea-pigs in 3 cases out of 29 when acid-fast bacteria were present in primary foci of children. Since the introduction of chemotherapy, observations like these have become more frequent. On examining 175 streptomycin-treated cases, D'Esopo (1948) obtained cultures in only 17 cases, though acid-fast bacteria were present in the sputum of 130 of the patients. In the discussion organized by the Trudeau Society, Steenken (1953) suggested that non-cultivable acid-fast bacteria may often regain their viability under favourable conditions. Bernstein and Steenken (1954) studied the effects of bovine albumin on tubercle bacteria of reduced viability with a negative result. Hobby (1954) applied two-phase cultivation first inoculating liquid medium and subsequently solid medium, and she observed the cultures over 9—12 months. From 9 out of the 15 cases tested, cultures were only obtained by this technique. Medlar (1956) and Wayne (1960) investigated materials obtained from lung resection. Wayne (1960), Murohashi et al. (1960) applied Hobby's method and examined the supposed stimulating effect of pyruvic acid, phthiocol, etc. None of these methods gave better results than the usual cultivation and animal experiments. Colletsos (1959, 1960) applying media containing simian liver extract, gelatin and rare elements exerting oligodynamic effect, often isolated tubercle bacteria which failed to grow under the usually applied conditions. Colletsos has spoken of the re-animation of bacteria with reduced viability.

As to the reactivation of bacteria the viability of which has been damaged by chemical and/or physical effects, it is known that micro-organisms inactivated with $HgCl_2$ may become viable again when treated with H_2S

(Topley and Wilson (1955). This is because the mercury that had penetrated the cell forms an insoluble precipitate with the sulphur and thus loses its deleterious effect. Flett et al. (1945), Jacobs (1945), Heinmets et al. (1954) succeeded in cultivating bacteria that had been killed with antiseptics and heat in media enriched with the metabolites of the Krebs cycle. They studied also the reactivation of UV-inactivated bacteria, based primarily on the observations of Kelner (1949). On the other hand, there are observations which fail to confirm the results of Flett and Heinmets. Tilley (1948), Garvie (1955) and Chambers (1957) suggested that the above injuries kill less than 100% of the bacterial population, and the small number of still viable bacteria will multiply, i. e. no true reactivation occurs.

Materials and Methods

Attempts were made to reactivate phenol-, heat-, or acetone-injured mycobacteria by consecutive passages in mice and under various growth conditions.

1. *Mycobacterium strains. M. tuberculosis* strain H_{37} Rv, BCG and 115 (Weissfeiler); *M. avium* Kirchberg; chromogenic atypical mycobacteria, strains No. 918, 919 and 1118, each isolated from patients.

2. *Phenol effect.* The strains were cultured in Dubos medium; 14-day cultures were diluted ten times and phenol was added to obtain 2% phenol concentration. After 48 hours the suspension was centrifuged and the bacteria were washed twice with phosphate buffer containing 0.01% Tween 80, (Dubos 1953).

3. *Heat effect.* Cultures were centrifuged, resuspended in phosphate-buffered solution in test tubes and heated for 60 minutes with agitation at 100°C in a water bath.

4. *Acetone effect.* Acetone-suspended culture was extracted by continuous agitation for 1 hour, then kept at 37°C for 1 or 24 hours in a glass-stoppered flask. Finally the acetone was removed and the suspension was washed with buffer.

5. *Animal passages.* New-born mice were inoculated with mycobacteria. Litter-mates (6—8) were inoculated with the same suspension, 5 mg subcutaneously or intraperitoneally. The animals were killed after 10—14 days and suspensions were made separately from the organs of each mouse. The cultures showing microscopic growth which was observed in smears were subjected to a subcutaneous passage in white mice of 10—15 g body weight. These mice were killed after 45—48 days and cultivation of their organs was attempted.

6. *Cultures.* As controls, haemolyzed blood, Dubos medium and Löwenstein—Jensen medium were inoculated with bacteria. Part of the organs of the animals were treated with 3% H_2SO_4, then washed twice with phosphate buffer (pH7) or, omitting sulphuric acid treatment, were washed with mixture of 2% bovine albumin and in 0.2% sodium pyruvate in phosphate buffer at pH7.0 under sterile conditions. In several cases organs were incubated for 1—24 hours in a mixture of the tricarboxylic acids of the Krebs cycle (Heinmets), then inoculated on to the above three media. In a few cases the tissue culture medium Parker's No. 199 was applied without antibiotics; 30, 45 and 90 day liquid medium cultures were centrifuged the sediment was examined under the microscope and after addition of the mixture of the tricarboxylic acids cultured on Löwenstein—Jensen medium, which was then observed for 3—4 months.

7. *Control experiments in guinea-pigs.* After treatment with phenol, heat or acetone, the suspensions of virulent tubercle bacteria were inoculated into guinea-pigs to control them for viable bacteria. The animals were killed after 1—3 months and passage was made in several cases from the pus formed at the site of inoculation, from the regional lymph node and from the spleen. Thirty guinea pigs were used altogether.

8. *Control experiments in albino mice.* Cultures were prepared from non-inoculated new-born and adult mice to control spontaneous infection with mycobacteria.

9. *Examination of the isolated strains.* In addition to microscopic examination, the growth at different temperatures, resistance to INH, streptomycin and PAS in Löwenstein medium and several biochemical properties of the strains (such as the niacine reaction, amidase spectrum, nitrate-reductase, lipase, arylsulphatase and catalase activities and neutral-red reaction) were examined. Virulence was examined by inoculating 1.0 mg into each of 20 mice intravenously and 1.0 mg into guinea-pigs subcutaneously. The immunogenicity of the strains was examined by inoculating 10 guinea-pigs with 1 mg each and re-infecting them 4 weeks later with 0.00001 mg of the virulent tubercle bacterium. The severity of the resulting illness was controlled after a further two months and expressed with an index.

Experimental Results

Two series of experiment were carried out. In the *first series* (Table I) the effects of heating at 100° C, of phenol and of acetone on strains H 37 R_v, M. avium, BCG, № 115, 919, 1118 were examined in 10, 10 and 1 experiments in 57, 63 and 5 mice, rescpectively (125 mice altogether). Twenty-one untrea-

TABLE I

Experiments on new-born mice

Damaging agent time	No. of experiments	No. of mice	Micro-scopic growth	Positive culture (from Strain)
100° 1 h	10	57	5	2 (919, 1118)
Phenol 2% 48 h	10	63	5	2 (avium, 115)
Aceton	1	5	—	1 (BCG)
Control	5	21	—	—
Total	26	146	10	5

ted mice from five litters served as controls. Neither microscopic nor subculturable growth could be observed in the cultures prepared from the control mice.

From the organs of mice inoculated with the heat-treated pigment-producing atypical strains No. 919 and 1118, white colonies grew out from which S-type cultures were obtained. From mice inoculated with phenol-treated suspensions of *M. avium* and strain 115 non-pigment-producing cultures forming smooth colonies were obtained. From the 21 experiments white cultures of the S-type grew out in 5 cases. Microscopically demonstrable growth was much more frequently observed; in these preparations acid-fast bacteria aggregated in the characteristic cord form were seen. Such microscopic growth was observed in 10 cases, i.e. in half of the experiments.

Second series of experiments (Table II). Two strains, viz. $H_{37}Rv$ and the pigment-producing strain No. 918 were examined. The former have never given positive results in earlier experiments. Sixteen experiments were carried out in 130 new-born mice, and 3 experiments in 40 guinea-pigs. For second passage of the $H_{37}Rv$ strain, 199 albino mice were used. The strain No. 918 was not passaged. The following results were achieved. We succeeded in re-isolating acid-fast mycobacteria in six experiments; in three of the positive cases the mice had been inoculated with the injured $H_{37}Rv$ strain, and in the other three cases with the injured strain No. 918. From the former four strains were obtained, all from the second mouse passage. Five to six months (156—183 days) had elapsed from the beginning of the experiments till the appearance of cultures. From the mice inoculated with injured bacteria of the strain No. 918, three strains were re-isolated, each without second passage. Appearance of the cultures needed 90—115 days, i.e. the relative incidence of positive cultures was higher and the growth of cultures was faster.

Without animal passage only one experiment, with the heat-treated strain No. 918, yielded culture of mycobacterium. The growth was observed after three subcultures, on the 198th day.

TABLE II

Passage of mycobacteria damaged by phenol, heat or acetone in new-born mice

Damaging agent	Strains	No. of experiments of strains obtained: new-born mice	No. of experiments of strains obtained: guinea pigs	No. of animals N/b	No. of animals G.p	No. of cultures and interval up to growth (days)		Succesive steps leading to isolation
	H 37 Rv	3/2	1/0	21	10	I. 216	175	N/b→Hb→Y→ L
						II. 218	156	N/b→Hb→Y→ L
						219	176	N/b→Hb→Y→Hb→L
100°C—60'	Control in vitro	4/0		—	—	—	—	
	918	3/1		22	—	229	99	N/b→DL
	Control in vit.	3/1				C	198	H/b→L/Hb→L
	H 37 Rv	6/1	1/0	40	23	205	183	N/b→199→ Y→D→L
	Control in vit.	7/0				—	—	
2% phenol 48h	918	1/1		16	—	232	104	N/b→ D→ L
						233	160	N/b→Hb→L/HbL
						234	165	N/b→Hb→L/HbL
	Control in vit.	1/0				—	—	
Aceton 24h	H 37 Rv	—	1/0	—	7			
	918	3/1	—	31	—	230	115	N/b→D→L
	Control in vit.	3/0						
Total	H 37 Rv + 918	16/6	3/0	130	40			
	Control in vit.	18/1	3/0					

Number of passage-animals:

I. New-born mice = 130
II. Young ,, = 198
III. Guinea-pigs = 40

Legend

N/b = New-born mice
Y = Young ,,
Hb = Haemolised blood medium
L = Löwenstein medium
D = Dubos ,,
199 = Synthetic ,,

The four strains originating from the $H_{37}Rv$ strain and the four obtained from strain No. 918 were subjected to investigations (Table III). Each has proved to be an S-variant, each propagated more rapidly than the original strain $H_{37}Rv$ and No. 918 and neither produced pigment. Most of the strains are resistant to INH, streptomycin, PAS and thiosemicarbazone (TbI). Only strain No. 205 proved to be sensitive to streptomycin and No. 230 to both streptomycin and INH. The mouse pathogenicity of the strains

TABLE III

Properties of the isolated strains

Damaged strains	Strains obtained following passage	Variant	Growth (days) at		Growth on media containing												Pathological changes in mice	Immunogenicity against tuberculosis (index)
					Streptomycin			INH			PAS			Tbl				
			24° C	37° C	5 γ	10 γ	50 γ	5 γ	10 γ	50 γ	5 γ	10 γ	50 γ	5 γ	10 γ	50 γ		
H37 Rv	216	S	4	3	+	±	−	+	+	−	+	+	+	+	+	+	+	10.4
	218	S	10	7	+	+	+	+	+	+	−	−	−	+	+	+	+	5.5
	219	S	7	4	+	+	−	+	±	−	+	+	+	+	+	+	+++	9.8
	205	S R	10	7	−	−	−	+	+	−	+	+	+	+	+	+	−	11.1
918	229	S non-pigm.	7	5	+	+	±	+	+	±	+	+	+	+	+	+	+	5.4
	230	S non-pigm.	7	5	+	+	−	+	±	−	+	+	+	+	+	+	++	9.4
	232	S non-pigm.	7	5	+	+	−	+	+	+	+	+	+	+	+	+	±	7.7
	Control C (918 100° 60)	S non-pigm.	10	7	+	+	±	+	+	+	+	+	±	+	+	+	Control	→ 11.0
Original 918		S pigm.	14	10	−	−	−	+	+	+	+	±	−	+	+	+	−	

was variable. Strain No. 219 proved to be the most virulent. Two strains, viz. No.218 originating from $H_{37}Rv$ and 229 originating from strain No. 918 were immunogenic against tuberculosis. As to the biochemical properties of the strains, nitratreductase and arylsulphatase were produced by all of them, whereas their amidase spectra were different; four strains possessed practically no amidase; strain No. 205 was urease-, nicotinamidase- and pyrazinamidase-positive.

Discussion

It has been shown in the present studies that passage in new-born mice and subsequent cultivation experiments in various media of heat-, phenol- or acetone-injured mycobacteria may result in isolation of mycobacteria different from the original strains in several characteristics. The doubt may arise that the re-isolated strains might originate from mycobacteria occurring spontaneously in mice. There are only three publications available in the literature on isolation of mycobacteria from albino mice (de Yong (1903), Hemmert—Halswick (1934) and Vischer (1954)) suggesting that spontaneous infection of albino mice with mycobacteria occurs rarely. From the 21 control mice we failed either to demonstrate microscopic growth or to obtain cultures, suggesting that our experimental animals had not been infected spontaneously by mycobacteria. The fact that no mycobacterium could be re-isolated directly from the new born mice inoculated with strain $H_{37}Rv$, but only after a passage in adult mice, while re-isolation of the strain No. 918 was successful from new-born mice directly also support the view that the mice had not been spontaneously infected. A control experiment initiated with strain No. 918 yielded a culture (strain C) without any passage in animals. However, this growth was only observable in the medium containing haemolyzed blood as late as after 198 days. This observation, nevertheless, indicates that strain No. 918 is more viable, and is able to reactivate even *in vitro*.

The re-isolated strains proved to be different from the parent strains in several characteristics, but they do not agree with the properties of known saprophytic mycobacteria, the latter showing much faster growth.

We suppose that the injuries applied failed to kill every individuals of the mycobacterium populations irreversibly and that there are favourable conditions in the cells of the new- born animal for the reactivation or regeneration of the injured bacteria. It is well known that, due to their high lipid content, mycobacteria are much more resistant than other bacteria. The re-activated mycobacterium strains, owing to profound genetic changes,

differ in several characteristics from the parent strains, suggesting that each of acetone, heat and phenol may act as mutagenic agent and at the same time exert a selective effect. Presumably the bacterial cells that are able to resist the damaging effect undergo mutations and can only multiply when more resistant mutants arise.

Summary

Mycobacteria injured in viability by phenol, heat or acetone may be re-activated in new born mice. The reactivated bacteria may grow after passages on different media. Atypical, pigment-producing strains are more suitable for such experiments than the virulent tubercle bacterium.

The strains re-isolated after reactivation, owing to profound genetic changes, differ from the original strains in several characteristics.

We failed to observe spontaneous occurrence of saprophytic or atypical mycobacteria in the albino mice used in the present experiments.

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IMMUNOLOGICAL INVESTIGATIONS WITH ENZYME (PHOSPHORYLASE) ANTIGEN*

by

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FROM immunological aspects, enzymes constitute a special group of antigens, in which the antigen possesses a special function: enzymic activity. The use of enzymes in the experimental research of immunity has several advantages: 1. methodologically: due to their enzymic activity enzymes are, soto say, marked which makes it possible to determine their amount in a complex mixture specifically and in a sensitive way; 2. theoreticaly: the study of the interaction between an enzyme and the corresponding antibody serves as an approach to the interpretation of the mechanism of action of both antibodies and enzymes. The enzyme-antibody-substrate ensemble is a three-component system which may stand for a model, due to its relative simplicity, for the study of the interactions of a more complicated system as toxin, antitoxin and organism. In the latter case, if toxin is compared to an enzyme, the host organism might be regarded as substrate. The investigation of the immunology and immunochemistry of enzymes may be fruitful from another aspect, too, i.e. if antibodies are applied as selective enzyme inhibitors due to their specificity.

In this lecture I give a brief account of the work carried out in our laboratory on the antigenic properties of phosphorylase.

One motive which has prompted us to study enzyme antigens in detail was a practical one, viz. the fact that the microquantitative determination of antibodies is a question far from being solved. At present, we have no method which would thoroughly fulfil all the requirements of antibody assay.

However, nowadays there has been an increasing claim for antibody determination methods which fulfil the main criteria of scientific exactness, i.e. proper sensitivity, quantitative character, specificity and good reproducibility. Moreover, even other demands have been raised, namely the

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can be converted into the dimeric form in the presence of Mg and 5'-AMP and in this state it can well be crystallized. This method proved to be easily search for suitable assay methods of antibodies in organ homogenates or tissue homogenates, for the study of sessile antibodies.

We thought that to fulfil these demands, at least partially, an enzyme-neutralization antibody determination will prove to be suitable.

An other interesting aspect of enzyme-antibody interactions was the investigation of the mechanism by which the antibody inhibit enzyme action and the specificity of the antibodies produced possess towards enzymes of other animal species and organs.

That phosphorylase has been adopted for our studies is due to several circumstances. First, an enzyme had to be chosen against which neutralizing antibodies are formed and which can be prepared possibly in pure form and its activity can be assayed simply and accurately. We have tried several enzymes: crystalline catalase, crystalline aldolase, myosin ATP-ase and cholinesterase, muscle true cholinesterase, but against none of these are neutralizing antibodies formed. Finally, muscle phosphorylase proved to be suitable for our purposes, therefore, most of our studies are concerned with this enzyme. The antigenic property of muscle phosphorylase was first described by us (Jókay et al. 1958). Our work carried out so far in this field can be divided into three categories: 1. preparation and purification of antigen and antibody; 2. the mechanism and specificity of the phosphorylase-antiphosphorylase reaction; 3. determination of circulating and sessile antibodies based upon their inhibitory effect on enzyme activity.

1. First we used Ph-a* prepared from rabbit muscle, according to Cori (Illingworth and Cori 1953), as enzyme preparation. This is an SH-enzyme of nearly 500,000 molecular weight and it is active also in the absence of 5'-AMP. On the effect of PR-enzyme this enzyme is converted to phosphorylase-b which has a molecular weight half that of Ph-a and exhibits activity only in the presence of 5'-AMP. Its activity can be assayed in the simplest way on the basis of inorganic phosphate liberated from glucose-l-phosphate in the presence of glycogen. The original preparation method of Ph-a, however, proved to be unsatisfactory, and one reason for this was the incomplete removal of PR-enzyme. A few years later Fischer and Krebs (1958) elaborated the crystallization of phosphorylase-b, by demonstrating that phospho-

* Abbreviations: Ph-a, phosphorylase-a: Ph, phosphorylase-b; PR-enzyme, phosphorylase-rupturing enzyme; AP, antiphosphorylase; 5'-AMP, adenosine-5' monophosphate; G-1-P, glucose-1-phosphate; GL, glycogen; EDTA, ethylenediamine tetraacetate.

rylase-b elaborated by us so far, for the purification of rooster Ph. By this method, reproducible by us and, moreover, it had a considerably higher yield than the former one. Immunization experiments on roosters were carried out with this antigen. Since muscle phosphorylase has class-specificity as shown by our studies, we attempted to prepare purified phosphorylase from roosters' breast muscle. As Cori's method for the purification of rabbit muscle Ph-a, when applied to rooster muscle, had the disadvantages of poor reproducibility and rather low yield, we tried to prepare phosphorylase-b from rooster muscle. The method for rabbit muscle Ph described by Krebs and Fischer, however, could not be applied to rooster muscle Ph at all, and after several attempts it became clear that also the chemical properties of the rooster Ph differ considerably from those of rabbit Ph. Because of this, a new method of purification had to be developed.

The main steps of this purification procedure are shown in Table I.

TABLE I

Preparation of phosphorylase-b

Rabbit skeletal muscle	Rooster breast muscle
(Fischer and Krebs)	
Watery muscle extract	Watery muscle extract
↓	↓
dialyzis	Fractionation with Rivanol
↓	↓
Removing of isoelectric precipitate at pH 6.0	Elution of Ph. from precipitate by a cation-exchange resin
↓	↓
Precipitation by $(NH_4)_2SO_4$	Precipitation of phosphorylase at 0° with Mg-acetate
↓	
Dialysis of dissolved precipitate	
↓	
Heat-treatment at alkaline pH	
↓	
Crystallization with Mg—5'—AMP	

The first colum shows the main steps of the preparation of rabbit muscle Ph according to Fischer and Krebs. By this method homogeneous, crystalline Ph can be obtained with good yield. The second column shows method

electrophoretically homogeneous, in the ultracentrifuge 80% pure rooster muscle Ph can be obtained, but crystallization has not yet been achieved. Nevertheless, the purity of this preparation is sufficient for the immunization of animals, and enables us to study the neutralizing antibodies produced.

The main adavantage of this preparation method is its rapidity as there is no ammonium sulphate precipitation and prolonged dialysis. While according to Fischer's method it takes about 5—7 days to prepare twice recrystallized enzyme, this preparation can be accomplished within 1—2 days. It might be worth mentioning that, according to our knowledge, rivanol-fractionation for enzyme purification has been applied first by us, a new methodical procedure which may be of interest to those concerned with preparative protein purification.

For this reason a few more details of rivanol-fractionation are given here. Rivanol, an acridine derivative, was first applied to the purification of human γ-globulin by Horejsi and Smetana (1956), when γ-globulin remained in the solution while other serum proteins precipitated. This procedure did not work, however, when we tried to apply it to the purification of rooster and rabbit γ-globulin, but the possibility has been raised that rivanol could be used to the fractionation of proteins. Rivanol precipitates proteins in the form of a poorly soluble complex, but the question of elution was not solved. After acidification the complex is dissolved and rivanol can be removed by adsorption on charcoal. This procedure seemed to be simple, but such a sensitive enzyme as Ph is denatured on acidification. Therefore, we applied a cation exchange resin, IRC—50, in NH_4 cycle, which replaces rivanol in the rivanol-Ph complex by NH_4. In this way Ph is solubilized and the pH is not altered considerably. A great advantage of rivanol fractionation is that it can be performed at room temparature and rivanol itself does not damage even such a sensitive enzyme as Ph, since phosphorylase activity can be recovered in 100% from the precipitate by added albumin or whole serum. Thus, the removal of rivanol can be achieved rapidly without dialysis, and in principle an electrophoretically homogeneous fraction is obtained as rivanol precipitates macromolecules roughly in the order of their net charge. Thus, e.g. from the serum albumin precipitates first and γ-globulin last. After this treatment of Ph a salt-poor and concentrated protein fraction is obtained. As compared with ammonium sulphate fractionation both concentration and purification were better by this method. On the basis of these advantages rivanol can be recommended for the fractionation of other sensitive macromulecules, too, where purification and concentration is necessary.

In the following a few data of the purification of rooster AP will be presented. In general, antibodies can be obtained from the immune serum in two ways:

1. by the physicochemical fractionation of antiserum;
2. by specific immunochemical methods.

In the latter case the washed specific antigen-antibody complex is dissociated and the antibody is separated from the antigen. To attain this several

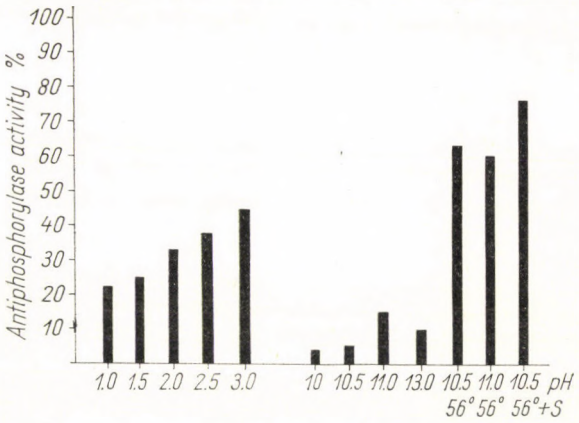

FIG. 1. Recovery of antiphosphorylase-activity from secific precipitate

ways can be followed, but it should be noted that the procedure depends in every case on the special physicochemical properties of the given antigen and, naturally, the antibody. This concerns, above all, the separation of antibody from the dissociated antigen-antibody complex. Although the difficulty of separation is partly eliminated by the use of specific immunosorbents, the preparation of these immunosorbents may be complicated especially in case of labile antigens. For the purification of antibodies produced in roosters against rabbit muscle Ph, the recovery of antibodies from the specific precipitate appeared to be a simple method, since the stability of the homologous antigen, compared with that of the corresponding antibodies, proved to be considerably lower. This made it possible to separate the denatured and precipitated antigen from the solubilized antibody in the dissociated antigen-antibody complex. Since there were no data on the stability of poultry antibodies, we examined first the stability of AP-antibodies formed in roosters at different temperatures, in acidic and alkaline buffers, and accordingly we attempted to isolate antibodies from the specific precipitate. The AP-activity recovered from the specific precipitate is shown in Fig. 1. Recovery is 20—40% at acidic pH, and it proved to be

best, if after dissolving the precipitate in glycine-sucrose buffer, pH 11, it was incubated at 56° C for 20 minutes. The dialyzed and concentrated AP, treated in the above way, retained its precipitating ability, and in the ultracentrifuge it proved to be a 90% pure antibody with a 7.5 S sedimentation constant (Jókay and Szabolcs).

2. In the following I shall discuss the mechanism by which the antibody produced against Ph inhibits the functioning of the enzyme. In general, antibodies formed against an enzyme may behave in different ways; they may 1. not influence, 2. inhibit, and 3. exceptionally, enhance the activity of the homologous enzyme. If the inhibitory effect of antibody prevails in the presence of excess antigen as well as at the equivalence point, i.e. it is independent of aggregate formation due to the precipitate, we deal an antibody designated by us as true antienzyme. To interprete the inhibitory effect of such antibodies two explanations have formerly been assumed:

1. the antibody is directed against the active centre of the enzyme;
2. it inhibits the functioning of the active centre via steric hindrance.

To examine the first possibility, the competition with substrate has usually been studied: the inhibition is competitive if at various substrate concentrations the degree of inhibition is also different, and when extrapolated to infinite substrate concentration, inhibition becomes zero; in case of non-competitive inhibition the extent of inhibition cannot be influenced by substrate concentration. In the original sense a competitive inhibition means that the inhibitor substance is bound to the same group as the substrate, while in case of non-competitive inhibition to another site. Actually, the situation is more complicated (Keleti 1964). A reaction may give non-competitive kinetics also when the inhibitor and substrate are bound to the same site but the binding of inhibitor is much stronger than that of the substrate. It appears that, at least partially, the inhibition by AP is a very similar one (Michaelides et al. (1964). In case of AP the extent of inhibition cannot be influenced appreciably by the variation of G—1—P or 5'-AMP concentration, thus, the inhibition is of non-competitive type. However, if the reaction is performed in the presence of Mg^{++}, which increases the binding of 5'-AMP four-fold to the enzyme, the inhibitory effect of AP can be influenced by 5'-AMP concentration and an inhibition of mixed type is obtained, where both V_{max} and K_m are changed (Fig. 2). Consequently, the antibody influences primarily the binding of activator; this antagonism, however, is not purely of competitive character.

In the course of investigations on the mechanism of action of antienzymes, more and more observations have accumulated which could not be completely interpreted in terms of the previously used concepts, e.g. an antibody

may inhibit also if it binds to a site remote from the active centre of the enzyme. Observations of this character can, however, be well explained if the enzyme molecule and its active centre are not regarded as a rigid immobile framework, but as a structure certain parts of which are in continuous movement related to one another and may assume various conformations depending on the circumstances.

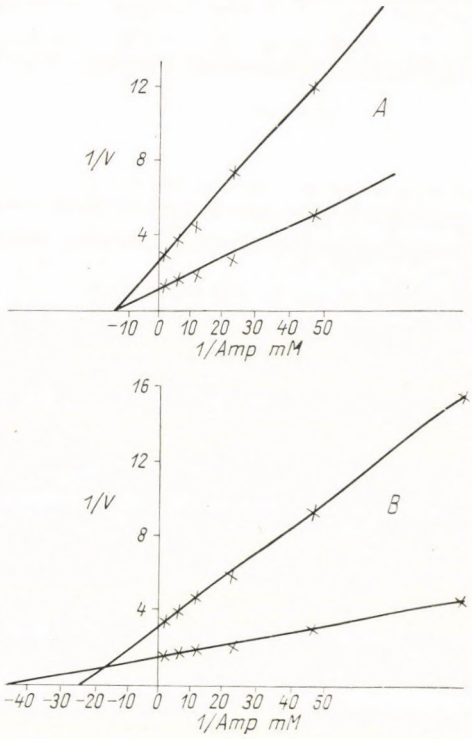

FIG. 2. Reciprocal velocity of phosphorylase-activity as a function of the reciprocal 5'-AMP concentration, in the presence and absence of antiphosphorylase. A = in the presence of EDTA; B = in the presence of 10 mM Mg-acetate

Recently, several theories have been proposed for the more precise interpretation of the mechanism of enzyme action, which have their common basis in the conformational changes of enzymes. One of them is Koshland's "induced fit" theory (1958), according to which substrate induces conformational alterations in the enzyme on its binding to the enzyme molecule. The other theory came from Monod et al. (1963) by the postulate of so-called allosteric enzymes. With these enzymes certain enzyme activators may act as follows: when bound to the enzyme these activators serve as

a rigid structure and would stiffen the structure of enzyme, or of its active centre, in a conformational form more suitable for the binding of substrate. In this way these activators affect the active centre through influencing the form of the enzyme without direct binding to the active centre. The negative allosteric effectors may operate by preventing the binding of activators or by the alteration of the steric structure of the active centre mediated through the protein framework.

Ph was one of the enzymes, the allosteric character of which was assumed first, and more recently also supported by experimental results, Helmreich and Cori 1964, Lowry et al, 1964 Madsen 1964, Ulman et al. 1964). According to Changeux et al. (1964), within an enzyme molecule the interaction between subunits held together by non-covalent binding forces can be altered by experimental conditions which results also in the change of conformation and kinetic parameters of the enzyme. As in Ph, SH-groups probably play a role in holding together the subunits (Madsen 1956, Madsen and Cori 1956, Madsen and Gurd 1956),we supposed that by blocking the SH-groups the structural changes of the enzyme induced by substrate and activator will become more prominent. Therefore, we studied the effect of substrate (glycogen, G—1—P) and activator (5'-AMP) on the inactivation of Ph by SH-reagents. In Fig. 3 the effect of substrate and activator can be

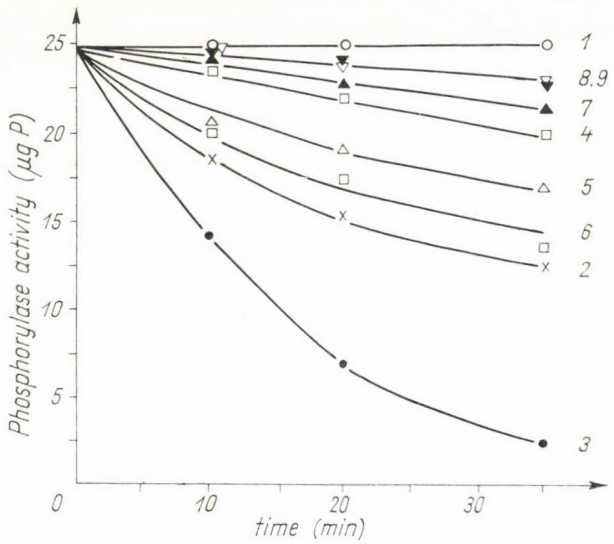

FIG. 3. The effect of substrate (glycogen, glucose-1-P) and activator (5'-AMP) on phosphorylase inactivation by PCMB as a function of time. Pre-incubation of phosphorylase-b with: 1 = control without PCMB; 2 = none; 3 = G-1-P; 4 = 5'-AMP; 5 = glycogen; 6 = glycogen + G-1-P; 7 = 5'-AMP + G-1-P; 8 = 5'-AMP + glycogen; 9 = 5'-AMP + glycogen + G-1-P

seen on the inactivation of Ph by PCMB: G—1—P increases, 5'-AMP decreases, while glycogen only slightly affects inactivation due to PCMB. Glycogen or 5'-AMP abolishes the sensitizing effect of G—1—P. 5'-AMP added together with G—1—P or glycogen protects more effectively than alone, although G—1—P itself has a sensitizing effect. In other words, G—1—P has qualitatively different effects depending upon whether 5'-AMP

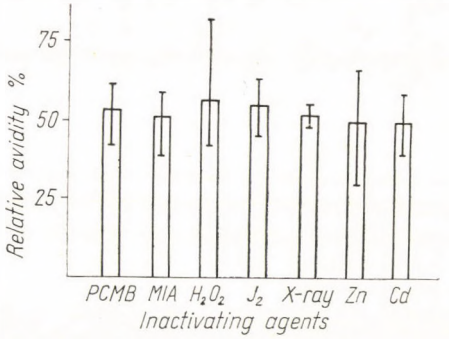

FIG. 4. Relative avidity of phosphorylases inactivated by SH-reacting agents

is present or not. Instead of a thorough discussion of these results, we might just draw the conclusion that these various effects can be interpreted in terms of conformational changes of the enzyme induced by substrate or activator. Actually, similar results have been obtained from experiments with another SH-reagent, N-ethylmaleimid, too (Jókay et al. 1965.)

While 5'-AMP protects the SH-groups especially in the presence of substrate, we have found that this effect is mutual: the intact state of SH-groups ensures the binding of 5'-AMP. SH-compound as cysteine and mercaptoethanol decrease the K_M of Ph towards 5'-AMP, while PCMB-treated Ph has a K_M greater than that of the native enzyme. It may be inferred that SH-groups, probably through the maintenance of subunit structure, participate also in the binding of enzyme activator (Jókay et al. 1965).

The possibility has been raised, therefore, that the inhibitory effect of AP could be explained by the blocking of enzyme SH-groups. To clarify this point, we examined the antibody-binding ability, i.e. the relative avidity of Ph inactivated by various SH-reacting agents as compared with active Ph control. The results are shown in Fig. 4. It can be seen that the relative avidity of Ph inactivated by SH-reagents (PCMB, iodoacetate), oxidizing agents (H_2O_2, I_2, and X-ray), or heavy metals (zink or cadmium) is about half that of active Ph. Furthermore, SH-compounds in high concentration can also moderate the inhibitory effect of AP. Since SH-compounds in such

high concentrations may exert non-specific effects too on the antibody, these latter results cannot be interpreted unequivocally as hapten inhibition. These experiments suggest that the SH-groups of Ph, either directly as antigen-determinant groups or indirectly by the maintenance of native subunit structure, play a role in the binding of Ph-antibody.

In Table II we demonstrate the effect of substrates and activator on the inhibitory effects of AP, if the former are added to the enzyme prior to the antibody. While glucose—1—P in itself renders the enzyme in a small degree more susceptible to the inhibitory effect of AP, glycogen protects the phosphorylase to a small extent. It is of interest to observe the combined effect of substrate components and activator. 5'-AMP alone has no significant influence in these experimental conditions upon the inhibition by antibody, but it exerts a protecting influence in the presence of glycogen and it sensitizes the effect of antibody if G—1—P is present. According to these, the effect of 5'-AMP differs qualitatively, depending on the circumstance whether the enzyme was previously pre-incubated with glycogen or G—1—P

TABLE II

Effect of pre-incubation of phosphorylase with substrate and activator on the inhibition of antiphosphorylase

Phosphorylase pre-incubated with	Inhibition (%)	Percentage increase (+) or decrease (—) of inhibition
None	59.5	0
5'—AMP	59.9	0
Glucose-1-P	62.5	+ 5
Glucose-1-P + 5'-AMP	71.2	+19.8
Glycogen	51.5	—13.4
Glycogen + 5'-AMP	41.5	—30.3
Glycogen + 5'-AMP + Glucose-1-P	39.5	—33.6

On the other hand, while G—1—P alone or in combination with 5'-AMP enhances the inhibitory effect of antibody, it exerts a protecting influence if it is added in combination with glycogen +5'-AMP. In such a case — when the enzymic reaction is running — the antibody has the least inhibitory effect.

Summarizing these investigations, we have established that pre incubation of the enzyme with various combinations of substrates (glycogen and

G—1—P) and activator influences the inactivation of the enzyme by the antibody in different manners. These data indicate that combinations of substrates and activator induce different conformational changes in the enzyme, and the extent of the inhibitory effect of the antibody is influenced by this induced structural alteration rather than by competition. This follows from the fact that by adding substrate or activator to the enzyme previously inhibited by AP, the extent of inhibition cannot be influenced by varying the concentration of these components. The fact that in the presence of Mg^{++} inhibition is of mixed type, i.e. the extent of inhibition can be diminished by increasing 5'-AMP concentration, can be explained by assuming an interaction between the binding sites of 5'-AMP and the antibody, possibly mediated through conformational changes. All these data suggest that the inhibitory effect of antibody depends upon the actual conformational state of the enzyme, or in other words, the antibody bound to the enzyme can inhibit the functioning of the active centre not only by steric hindrance but also by evoking conformational changes.

On the species-specificity and organ-specificity of Ph the following can be mentioned. Rooster antibodies produced against rabbit muscle Ph inhibit uniformly the muscle, diaphragm, and heart Ph, while thly do not inhibit liver Ph of other mammalian laboratory animals. (rat, mouse, guinea-pig). Antibodies produced in rabbit against rooster muscle Ph inhibit muscle Ph from other poultry species (duck, pigeon) very well, while they do not inhibit Ph from other mammals and liver Ph. Consequently, muscle Ph has pronounced class-specificity and organ-specificity, so that, as to antigenic properties, muscle-Ph-s within the individual classes are nearer to one another than Ph-s of different organs within the same animal, as e.g. liver and muscle Ph (Jókay etal. 1958). Naturally, this does not mean that within a class one cannot distinguish Ph-s of different species, but this is much more difficult (Yunis and Krebs 1962). The circumstance that an antigen might have class specificity should be especially taken into account when the animal species suitable for immunization is being adopted. While formerly rabbit was regarded as one of the classic objects for the production of immune sera, this concept nowadays loses its general character more and more, particularly in studies on mammalian antigens. According to our experience, poultry, roosters and hens, especially when certain mammalian antigens are studied, are often almost indispensable and, moreover, from several points of view they are even better antibody-producers than rabbits.

3. In the third part of my survey I should like to dwell on the conditions under which AP-antibodies can be determined quantitatively on the basis

of their inhibitory effect on enzyme activity. The following questions have been studied in this connection:

1. The significance of the Ph/AP ratio in the assay
   (a) conditions under which the inhibitory effect of AP serum is proportional to antibody content;
   (b) what relation can be detected between the inhibitory effect and antibody content of sera.
2. Sensitivity of AP-assay;
3. Elimination of non-specific inhibitory effects or their correction;
4. Heat-labile and heat-stable inhibitory effect of normal plasmas;
5. The time course of Ph-AP reaction;
6. Expression of AP-content in units;
7. Study of the primary and secondary immune response in roosters.

If the percentage inhibition is plotted as a function of the concentration of immuneserum, the extent of inhibition is directly proportional to the amount of AP up to about 66% relative inhibition, while above this value the curve levels out. On the other hand, if the inhibition values are registered as a function of enzyme concentration, using the same amount of immune sera, it is shown that in case of an appropriate antigen excess the absolute inhibition values are constant, independent of the amount of enzyme added.

From the point of view of AP-assay, it follows from the course of these curves that a ratio of antigen to antibody should be employed where the relative inhibition does not exceed 66%. It is more safe, however, to take this limit for 40%. Under these conditions inhibition values expressed in antiphosphorylase units are proportional to the antibody content (Jókay 1963).

It is an important aspect in enzymatic antibody determinations that the sensitivity of the method can be increased by decreasing the amount of enzyme-antigen used with the simultaneous prolongation of the time of activity assay. Table III shows that Ph-activity and AP-activity changes linearly with dilution and time, i.e. the product of multiplication of the amount of enzyme and of reaction time is constant. If 6-fold or 18-fold dilutions are made of the enzyme and antibody, and accordingly enzyme activity is measured for 60 or 180 minutes, respectively, instead of 10 minutes, the same Ph activity or inhibition values are obtained as in 10 minutes, though the amount of antienzyme measured is only 3.3 and 1.1 APU, respectively instead of 20. This means that while using the standard 10 minutes reaction time, we can detect at most 1 APU, but with 180 minutes

TABLE III

Proportionality of phosphorylase and antiphosphorylase activity with dilution and incubation time

Incubation time (min)	10	60	180
Dilution of Ph and AP	1 x	6 x	18 x
Ph activity (μg P)	56	55	55
Ph + AP activity (μg P)	36	35	35
Inhibition (μg P)	20	20	20
APU measured	20	3.3	1.1

reaction time the detection of an 18-times less amount, i.e. about 0.05 APU, is also feasible. This corresponds approximately to 0.001 μg of antibody-N.

While in case of hyperimmune sera, the nonspecific inhibitory effect of sera is negligible, when small amounts of antibody are measured it should be taken into account more carefully. Therefore, we have studied the nonspecific inhibitory effect of normal plasmas on the activity of rabbit muscle Ph. We distinguish heat-stable and heat-labile inhibitory effect of normal plasma at 95°C. While the heat-stable inhibitory substance of plasma proportionally decreases the activity of Ph, the heat-labile inhibitory substance binds stoichiometrically to Ph. This heat-labile inhibitory substance of normal rooster plasmas can be found in the globulin fraction precipitating at half-saturation with ammonium sulphate, and in AP-assay it cannot be distinguished from immune bodies; therefore, they may be regarded as normal antibodies. Normal rooster plasmas contain on average 0.81 APU/0.1 ml serum heat-labile inhibitory substance.

By studying the time course of Ph-AP reaction, we have shown that the reaction is rapidly completed, and even after 15 minutes of pre-incubation inhibition attains about 95% of that observed after 60 minutes of pre-incubation.

In Table IV the procedure of AP-assay is shown. In case of small amounts of antibody two samples are run of each antiserum, one of which is heat-treated at 95°C to inactivate the antibody. In addition, controls are run to each series with standard diluent. After the addition of properly diluted antigen, samples are pre-incubated for 20 minutes, then the substrate is added and the Ph activity is determined on the basis of inorganic phosphate liberated in the protein-free filtrate.

The inhibitory effect of AP-sera is expressed in "absolute units" which is independent of the heat-stable inhibitory effect of plasmas and is directly proportional to antibody content.

Afterwards we examined the amount of Ph required for an optimum primary and secondary immune response in roosters weighing about 1.8—2.0

TABLE IV

Performance of antiphosphorylase determination in plasma

S	I_n	I_h	Blind
0.2 ml standard diluent ∅	0.2 ml antiphosphorylase dilution ∅	0.2 ml 1'95°	(without incubation)

← 0.2 ml phosphorylase dilution →
← Pre-incubation 30°; 20' →
← 0.4 ml substrate →
← Incubation 30°; 10—60' →
← 3.2 ml trichloracetic acid 10% →
← centrifugation →
↳ 2.0 ml
1.0 ml H_2O
2.0 ml P-reagent

kg. Figure 5 shows that when three groups of roosters were immunized with 5, 10, and 20 mg of Ph, respectively, 10 mg of antigen proved to be optimal at the primary injection. To obtain secondary immune response, 10 mg of Ph was administered to the animals uniformly, whereupon a seven times greater antibody content was measured than after the first injection. It is noteworthy how rapidly the antibody content decreased after attaining maximum level (Jókay and Tóth 1966).

After having determined the assay conditions for circulating antibodies, we adapted this method to organ-homogenates for the quantitative determination of AP-antibodies bound to the cells. We studied various factors influencing AP-assay in organ homogenates: 1. the own Ph activity of homogenate; 2. the high P-content of homogenate; 3. the AP-inactivating effect of homogenate; 4. the non-specific inhibitory or activating effect of homogenate; 5. the heat-labile inhibitory effect of normal homogenates; 6. the recovery of AP from normal organ homogenates. Based upon these data we elaborated the determination of AP in organ homogenates, the principle of which is the following. The heat-labile inhibitory effect of organ homogenates of roosters, immunized with rabbit muscle Ph, on the activity of enzyme-antigen is determined and correction is made for the AP content of blood found in the organs (Jókay et al. 1966, Tóth and Jókay 1965).

The occurrence of sessile antibodies was then examined in immunized roosters in various organs. On the 5th day following the third antigen

administration samples were taken from blood and liver, under sterile conditions, then after 8—15 days the animals were bled and AP-content in the blood, liver, spleen, lungs, kidney, and lymph-nodes was assayed. The

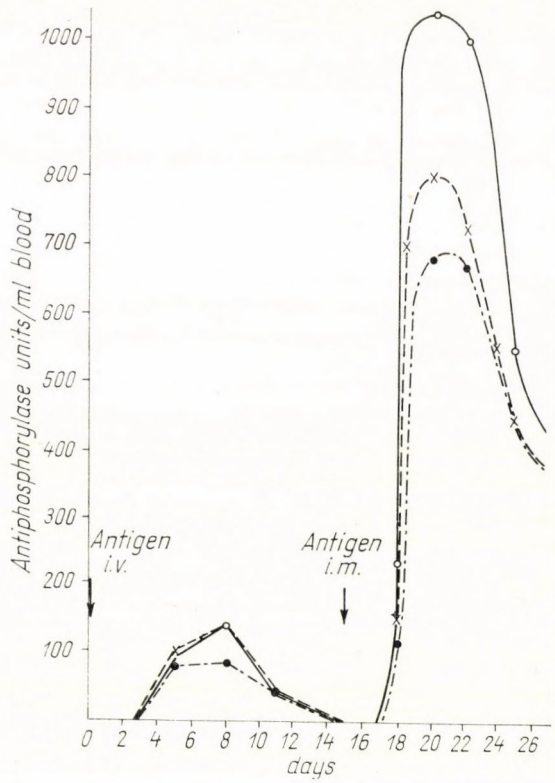

FIG. 5. Primary and secondary immune response in roosters to phosphorylase antigen;
●—·—·—·—● = 5 mg antigen;
o ——— o = 10 mg antigen;
x — — — — x = 20 mg antigen

results are shown in Table V. During the 8—15 day period the antibody content of both plasma and liver decreases considerably. In the antibody content of organs there are great individual differences, similar to plasma antibody content which can be partly attributed to the fact that we did not use animals of the same strain. If the ratio of liver tissue antibody content to circulating antibody content is calculated, it is found that this ratio is approximately the same before and after the 8—15-day period, although there is a 7-fold change in plasma antibody level. This suggests that sessile antibodies in the liver are passively deposited, depending on plasma anti-

TABLE V

Plasma- and cell-fixed antiphosphorylase values of the control and narcotized roosters before and after the narcotizing period

	Number of animals	Antibody content of plasma		Antybody content of liver		Antibody content of			
						Kidney	Spleen	Lung	Lymphn
		Test I	Test II	Test I	Test II	on the day of Test II			
		Antiphosphorylase-units (0.1g) wet-weight							
Control group average	11	210	32	12	2	8	9	9	6
Narcotized group average	10	244	24	16	8	7	6	13	13

body content, and thus, liver antibody content is to some extent the function of plasma antibody level. Among the other organs examined, lungs have perhaps the highest antibody content and livers have the lowest, but on average neither organ has a particularly high antibody content.

It should also be mentioned that in roosters the sessile antibody content of the organs is relatively very small compared with other mammalian laboratory animals. This may be due possibly to the higher body temperature and more intensive metabolism of roosters, at least to some extent. Furthermore, this might be the reason of the extremely rapid change in the number of circulating antibodies; after stimulation by antigen, rapid increase is followed by a rapid decrease. Probably it is not by chance that the antibody level is the lowest in the liver which is rich in enzymes, and is the highest in the lungs which is comparatively poor in enzymes and consequently might have a reduced metabolic rate, too.

In the foregoing I gave a brief account of conditions under which AP-antibodies can be determined on microquantitative scale on the basis of their inhibitory effect to enzyme activity.

On discussing the above delineated microquantitative antibody determination method, we can add the following remarks. For the study of primary immune response, *in vitro* antibody formation as well as for problems of the chemical structure of antibodies, first of all highly sensitive, simple, well reproducible quantitative antibody assay methods are required. It is also apparent from Marrack's (1963) survey that there is no universal antibody assay method at our disposal which would fulfil the maximum criteria of antibody determination in every respect. Although the modern precipitin reaction by Heidelberger, Kendal and Kabat measures quantitatively the total amount of precipitating antibodies which bind to the soluble antigen,

but owing to its low sensitivity it is not suitable for the determination of small amounts of antibodies. The other assay methods are based upon certain function of the antibodies. Of these the widely used passive haemagglutination test, though sufficiently sensitive, does not satisfy quantitative requirements and its reproducibility is also poor. A separate group is formed by special antibody determinations, by which antibody neutralizes certain marked functions of the antigen (toxin, virus, bacteriphage, enzyme). Medically and biologically these functional antibody determinations, based upon the neutralization of the biological function of the antigen, are of greater importance, while the determination of all antibodies (neutralizing + not neutralizing), e.g. on the basis of N-content assay, taking into account the great heterogeneity of immune globulins, is primarily of theoretical significance.

In experimental research on immunity, from the methodological point of view, enzyme-neutralizing antibodies should be first considered out of neutralizing antibodies, since a common advantage of enzymes over various toxins, viruses or bacteriophage particles is that they can be prepared in pure (homogeneous) form and can be assayed in a relatively simple, specific and sensitive way. Unfortunately, not all the enzymes can be used for enzyme-neutralizing antibody determination in experimental research on immunity. The main theoretical requirements of ideal antibody assay based upon the inhibition (neutralization) of enzymic activity can be summarized as follows:

1. the antibodies formed should be neutralizing ones, and should inhibit enzymic activity also in the presence of excess antigen, independent of precipitate formation (true antienzyme);
2. antibodies should be of relatively high avidity, to exert maximum inhibitory effect even at moderate antigen excess;
3. antigen should possibly be homogeneous, against which a well measurable primary immune response is obtained (strong or medium strong antigen);
4. the assay of enzymic activity should be simple, exact and well reproducible.

Only sporadic attempts of antibody determinations on the basis of enzyme neutralization can be found in the literature. One of the main reasons for this is the fact that, in general, there is no true antienzyme formation against enzymes. This can be explained in the first place by the following. In the course of evolution those groups (coenzymes) which are responsible for catalytic activity did not change or changed only slightly, otherwise catalytic activity would have been abolished; consequently, the antigen deter-

minant groups specific for species, organ, etc., developed primariliy in the carrier proteins of enzymes (apoenzymes) and the binding of these with immune globulins mostly does not completely cancel enzymic activity. The probability of production of neutralizing antibodies against an enzyme is the greater:

1. the greater the taxonomical difference between species serving as antigen sources and as immunized organisms, respectively;
2. the higher molecular weight has the substrate of the enzyme (e.g. a macromolecule);
3. if in the immunized organism there is no enzymic activity corresponding to the antigen (Cinader 1963).

Considering all these factors, rabbit muscle Ph belongs to the few enzymes against which true antienzymes are formed and the enzyme also fulfils the other criteria listed above.

Accorring to Kesztyüs and Gyulai (1954), prolonged barbiturate narcosis decreases the amount of circulating antibodies in rabbits. We employed the method described above, to study whether prolonged barbiturate narcosis influences the amount of circulating and sessile antibodies in immunized roosters.

We have established, using 10 narcotized and 10 control roosters, that prolonged barbiturate narcosis does not influence the rapid decrease of circulating antibodies in animals previously immunized, while the amount of sessile antibodies in the liver decreased at a lower rate in the narcotized group than in the controls. Barbiturate narcosis did not cause any detectable change in the level of sessile antibodies of other organs (Jókay et al. 1966).

In brief, I wanted to give this account of our investigations carried out so far on the Ph-AP system. In my opinion the microquantitative antibody assay method might claim wider interest, as the application of the method, due to its advantages, may promote the elucidation of certain immunological problems, even if the determination of an antibody of non-universal character is in question. Experimental research often employs tests, by the aid of which the effects of experimental influences can be measured in a simple, sensitive and quantitative way. The application of Ph as antigen appears to be suitable for such a test.

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RESULTS OF STUDIES ON ATTENUATED HUMAN TYPE ANTI-TUBERCULOSIS VACCINE STRAIN W 115

by

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CALMETTE an Guérin's BCG strain has been found very effective as a live antituberculosis vaccine. In order to increase the effectiveness of vaccination against tuberculosis, it is desirable to improve the methods of artificial immunization. Better results can primarily be obtained by applying an optimal BCG strain for vaccination. As considerable differences have been found in the immunogenic properties and residual virulence of BCG strains maintained in various laboratories, extensive research work is done in comparing BCG substains to select the best culture as a standard. The second task is the stabilization of BCG vaccine. This can be done by the lyophilization method elaborated by Weissfeiler and Leshinskaia (1937, 1940). Thirdly, the medium and the cultivation period yielding the most effective vaccine should be considered. In 1940 we showed that younger cultures possessed a higher immunogenic activity. Finally, it is very important to elaborate the most effective form in which the vaccine should be given: the dosage and the mode of administration (intracutaneous, percutaneous, or massive oral doses). This problem can be regarded as practically solved, since intracutaneous vaccination has proved to be a well-controllable and effective method.

The application of new, more effective vaccine strains. points the way to an improvement in vaccination against tuberculosis. In view of findings indicating that the BCG strain itself may be unstable, it is important to search for a reserve strain. Theoretically, it may be assumed that the BCG strain does not yield the best possible live vaccine. Few investigations have so far been carried out into this problem, with the exception of Well's Vole bacillus vaccine which has been widely studied especially in England and Czecho slovakia. These experiments showed negative results and so hopes about the Vole bacillus as a live vaccine have failed to be realized.

Our experiments carried out since 1932 in order to find a highly immunogenic attenuated vaccine strain, have led to studies on strain W 115. This strain was obtained from the blood of a patient suffering from pulmonary

tuberculosis by regenerating the filtrable form of the causative agent. Filtrable forms present in the blood were adsorbed to aluminum hydroxide gel and inoculated into guinea-pig. An attenuated tubercle bacterium, strain 115 was isolated from the animal. As for all live vaccines, also for strain 115 3 main properties are required: 1. The residual virulence of the strain should be at a degree which safely excludes the danger of progressive infection. Our experiments with guinea-pigs and *Macacus cynomolgus* monkeys, which had been inoculated with large doses of strain 115, never revealed the development of progressive tuberculotic lesions. In contrast, the animals showed slighter pathohistological alterations the later they were sacrificed. 2. The attenuated virulence of the strain should be stable, i.e. it should not increase under any circumstances. Such examinations were carried out on guinea-pigs. During a 4-year period 7 passages were carried out, both by direct transfer from the organs and by inoculating isolated passage cultures. These experiments have shown that the virulence of strain W 115 cannot be increased in animal passages. 3. High immunizing effect. The immunogenicity of strain W 115 was examined in 7 guinea-pig experiments and also in *Macacus rhesus* monkeys. In all experiments strain 115 yielded better results than the control BCG strain. A particularly definite high immunogenicity was shown in monkeys and, with substrain 115/9 obtained from strain 115 by selection, in guinea-pigs.

In addition, we have been able to obtain a strain resistant to 50 μg/ml INH. In immunogenicity this strain was equivalent with BCG. Šula, Šulova and Spurná have revealed similar results. This strain yielded good results in volunteer experiments, then in tuberculotic patients treated with combined INH + vaccine therapy in Tashkent.

As a result of these experiments strain W 115 was thoroughly studied by the following investigators: Dr. Jablokova (Tarassievitch Institute for Vaccine Control, Moscow), Dr. Govorov and Dr. Kassich (Ukranian Experimental Institute for Animal Health, Charkov), Dr. Inogamov (Institute of Microbiology, Tashkent), Dr. Šula (Czechoslovak National Institute for Tuberculosis, Prague), Dr. Stoyanov (Institute of Microbiology and Epidemiology, Sofia) and Dr. Földes (National Korányi Institute for Tuberculosis, Budapest). The importance of these investigations is underlined by the fact that the properties of strain 115 have been examined independently from the author and thus, the results are free from subjective factors. With the exception of the Charkov teams which have not yet summarized their results, participants of the co-operative study discussed the results at a round table conference in October 26, 1965 and reached agreement on further studies. Inogamov and his group have publishied their results ob-

tained on monkeys. The material presented and the standpoint of the conference are included in this publication.*

According to the decision of the Health Scientific Board of the Hungarian Ministry of Health, lyophilized ampoules of strain 115/9 will be provided for any investigator wishing to perform studies on this subject. In addition, on the basis of the decision of the Health Scientific Board, large-scale animal experiments will be performed to compare the residual virulence and immunogenicity of strain 115/9 and of strain BCG Paris 1102 in guinea-pigs and white mice.

The effectiveness of strain W 115 as a prophylactic live vaccine against tuberculosis should finally be estimated by comparison with the BCG vaccine in human beings. From theoretical considerations and from the experimental results obtained especially with monkeys, the aspects in this respect are promising as strain W 115 belongs to the human variety.

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* In debating the results of the conference, Dr. Sula did not wish to include as a dissenting opinion his negative standpoint expressed in his discussion as to the continuation of the investigations.

STUDIES ON THE RESIDUAL VIRULENCE OF THE STRAIN 115 OF WEISSFEILER

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Our most effective method for the prevention of tuberculosis is vaccination with living BCG vaccine, resulting in a relative specific immunity. In Hungary vaccination of new-born infants and revaccination of tuberculine-negative individuals every third year is obligatory. As a result of vaccination and other efforts, the morbidity of children has greatly diminished in the last few years. However, it is our further task to look for new vaccines more effective than BCG. We can tell whether a new vaccine is more effective than BCG, if 1. its residual virulence is not stronger than that of BCG, and 2. if its immunogenic effect is higher than that of BCG.

In this paper the results of our investigations regarding the residual virulence of the strain 115 of Weissfeiler are summarized.

Methods

Investigations have been carried out on guinea-pigs, albino rats and rabbits.

Experiments on Guinea-pigs

1. Ten guinea-pigs of the same sex and age were infected intraperitoneally with 10 mg wet weight of the BCG Paris strain and 10 with the 115 strain. The two strains under investigation were cultured in parallel on potato-media, and the suspensions used for the experiments were prepared also in parallel by the same methods from the 10-day old cultures. The guinea-pigs used were tested with 0.1 ml OT diluted to 1 : 100 by the Mantoux method. The body weights of the animals were controlled weekly, then 53 days after the injection they were killed by $CHCl_3$. The weights of lungs and spleens were taken, then the animals were studied by gross and pathohistological examination.

2. In the second series of the experiments on guinea-pigs, the animals were injected by the same method as in series 1, but the animals were sacrificed only 165 days after the injections.

Experiment on Rats

Ten albino rats of our own strain were given 10 mg of the BCG Paris strain, and 10 were given 10 mg of strain 115 intravenously. The suspensions were prepared from 20 day old Sauton cultures. The body weights were controlled weekly, and after 74 days the animals were sacrificed. The weights of lungs and of spleens were taken, and detailed pathological examinations were carried out.

Experiments on Rabbits

Five rabbits in each group were treated and controlled in the same manner as in the experiments with rats.

Results and Discussion

The Mantoux reactions of the guinea-pigs proved to be negative after 48 and 72 hours.

Figure 1 shows the average body weights of the guinea-pigs in experiment 1 in g-s, and Fig. 2 that in percentage of the starting body weights. Both figures show that the body weights of the guinea-pigs injected with BCG and strain 115, respectively, have increased in parallel. Table I. shows the average weights of spleens and lungs of the two groups of guinea-pigs with the quadratic deviations and the results of the statistical analysis performed

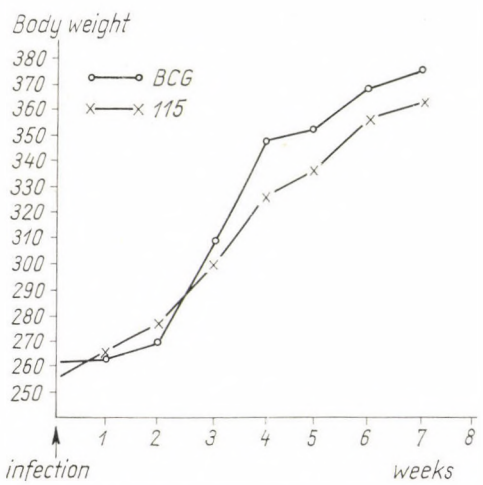

FIG. 1. Average body weights of the two groups of guinea-pigs in experiment 1

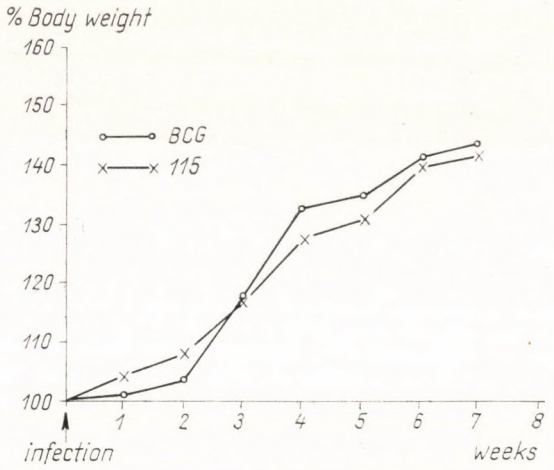

FIG. 2. Average body weights of the two groups of guinea-pigs in percentage of the starting average body weights (experiment 1)

with Student's "t" test. It can be seen that the average spleen weight of the guinea-pigs injected with BCG are somewhat higher than those injected with strain 115. In contrast, the average lung weights of the guinea-pigs injected with BCG are somewhat lower than those injected with strain 115. These differences proved to be insignificant statistically.

TABLE I

Study of the residual virulence of BCG and 115 strains in guinea-pigs (the experiment lasted 53 days)

Group	No. of animals	Average lung weights (g)	Statistical analysis	Average spleen weights (g)	Statistical analysis
I. BCG 10 mg i. p.	10	3.81±0.25	$t = 2.63$	0.77±0.30	$t = 1.69$
II. 115 10 mg i. p.	10	4.48±0.92	$0.02 > p > 0.01$	0.57±0.22	$0.1 < p < 0.2$

At post-mortem examination no tuberculous gross changes were found in the parenchymatous organs of the two groups of animals. However, pathological changes were found in the mesenteric lymph nodes. These lymph-nodes proved to be enlarged and filled with caseous necrotic masses. For example, the weight of the mesenteric lymph node paquet of animal No. 8 injected

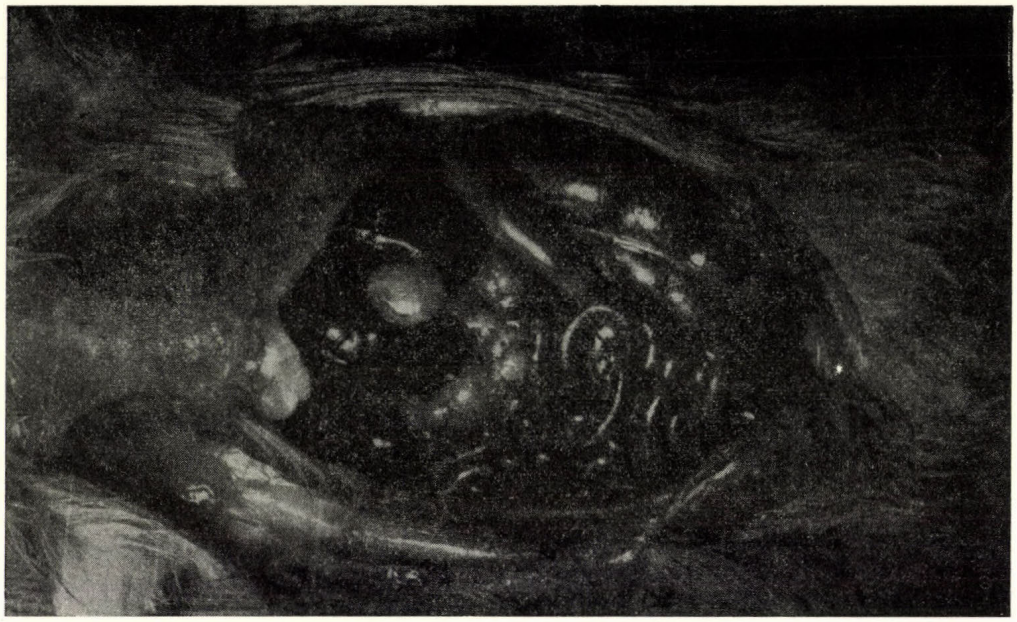

FIG. 3. Guinea-pig No. 8 injected with BCG

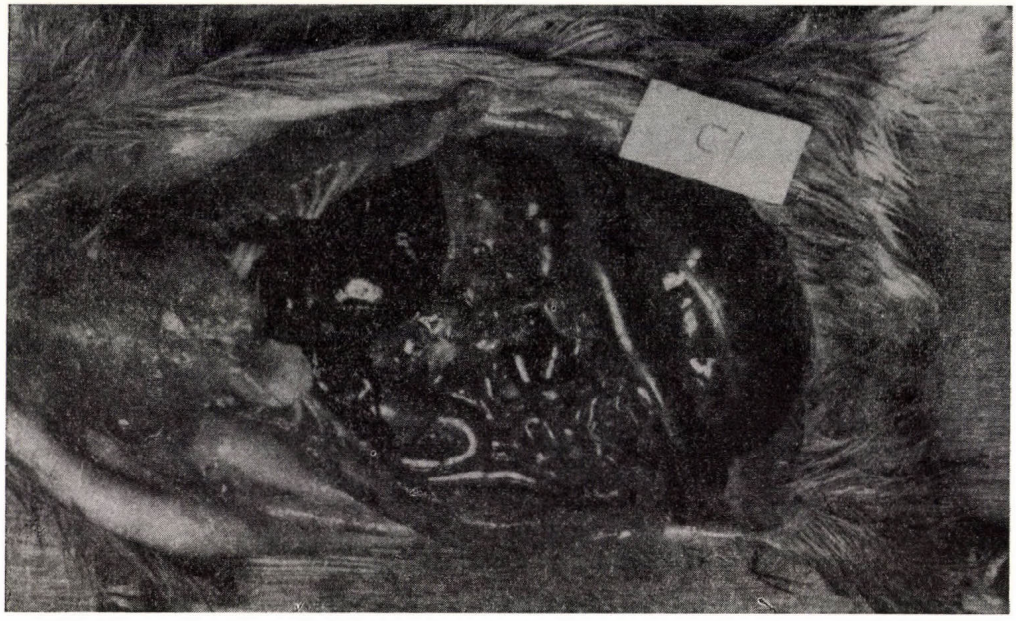

FIG. 4. Guinea-pig No. 15 injected with strain 115

with BCG was of 3.48 g (Fig. 3), and that of animal No. 15 injected with strain 115 was of 3.25 g (Fig. 4). Figures 3 and 4 show the gross pathological changes of these two guinea-pigs.

The histologic examination showed only mild changes in the lungs, livers and spleens. Thus, circumscribed small foci without necrosis and specific histological character were observable in the lungs and livers, and a follicular hyperplasia and a marked reticular character in the spleens. In the mesenteric lymph-nodes, grave changes with specific tuberculous character and central

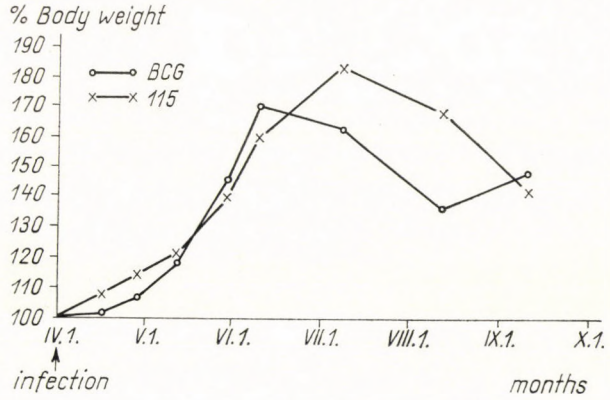

FIG. 5. Average body weights of the two groups of guinea-pigs in percentage of the starting average body weights (experiment 2)

necrosis were observed. A few giant cells of Langhans type were also seen in these lymph-nodes.

The second series of our experiments on guinea pigs lasted 165 days. Three animals of each group died of intercurrent diseases in the second half of the experiment. Figure 5 shows the average body weights of the animals of the two groups in percentages of the starting body weights. The average spleen weight of the guinea-pigs injected with BCG proved to be 1.21 g, and the average lung weight was 7.69 g. The corresponding values of the guinea-pigs injected with the strain 115 proved to be 1.41 g and 6.84 g, respectively. Thus, it can be stated that no significant difference between the two groups examined has been observed during this prolonged experiment. The same was found at the post-mortem and histological examinations. In both groups of guinea-pigs specific tuberculous changes with caseous necrosis were observed in the mesenteric lymph-nodes, but no such changes were found in the parenchymatous organs. Only a few circumscribed foci consisting of macrophage cells were found in the lungs (Figs 6 and 7).

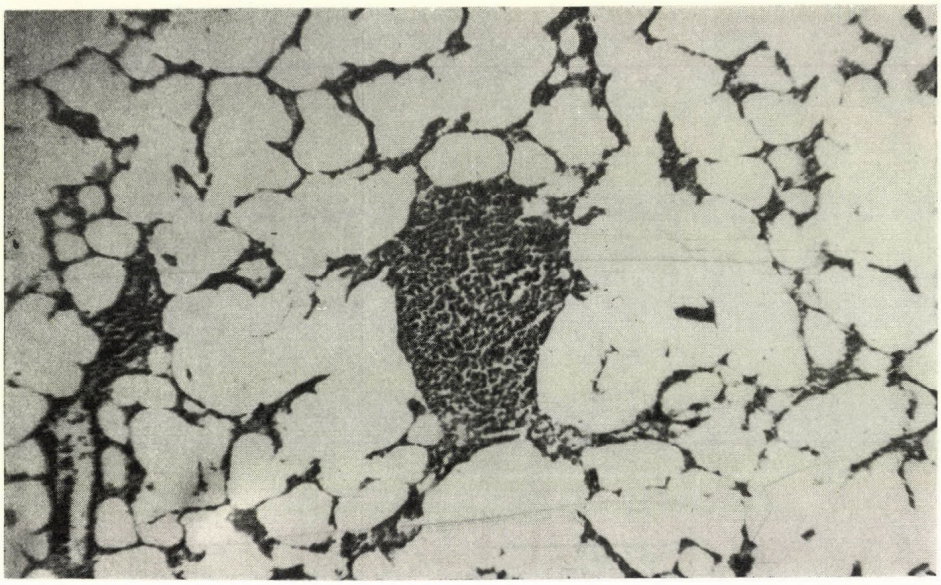

FIG. 6. Microscopic picture of the lung of a guinea-pig 165 days after injection of 10 mg BCG

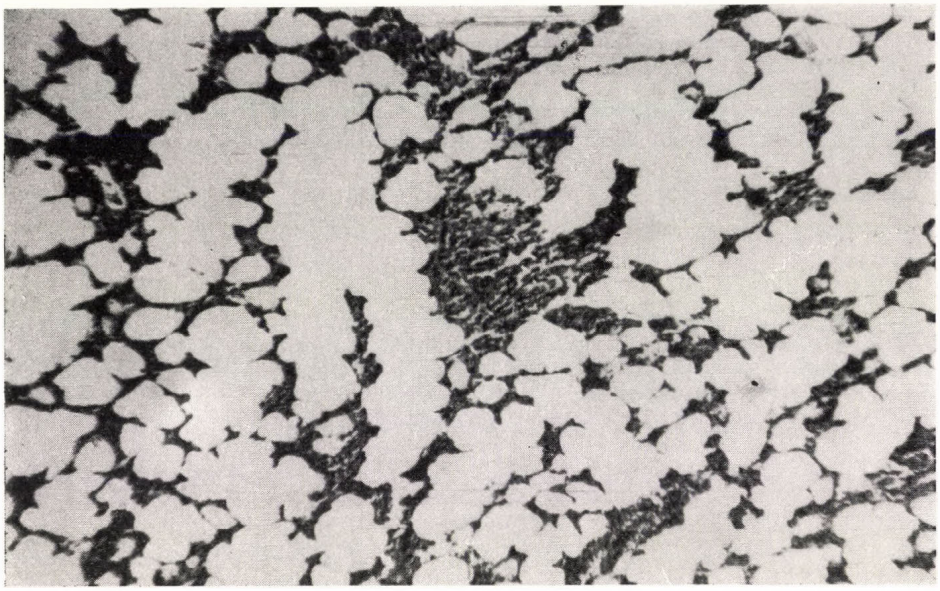

FIG. 7. Microscopic picture of the lung of a guinea-pig 165 days after injection of 10 mg strain 115

Figure 8 shows the curves of the body weights of the two groups of rats injected intravenously with BCG and strain 115, respectively. It can be seen that the two curves run in parallel. Table II shows the average weights of spleens and lungs of these two groups with the corresponding quadratic deviations and the results of the statistical analysis. It can be seen that the

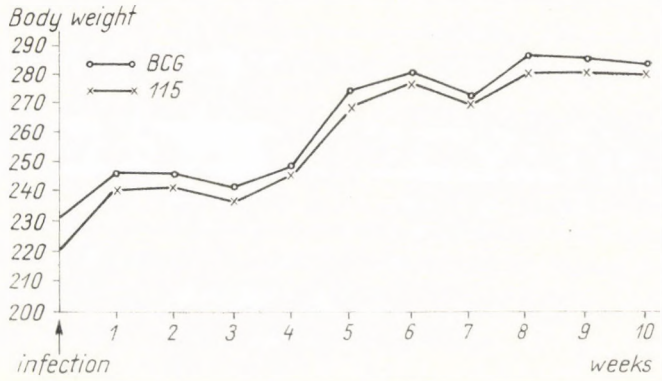

FIG. 8. Average body weights of the two groups of rats

average weights of the lungs and spleens of the rats injected with BCG are somewhat lower than those of the rats injected with strain 115. This difference, however, proved to be insignificant statistically. At the post-mortem examination of the animals of the two groups only a few small circumscribed foci consisting of macrophage cells were observed. It could not be stated that there was any difference between the two groups.

TABLE II

Study of the residual virulence of BCG and 115 strains in rats (the experiment lasted 74 days)

Group	No. of animals	Average lung weights (g)	Statistical analysis	Average spleen weights (g)	Statistical analysis
BCG 10 mg i. v.	10	2.34±0.29	$t = 2.47$	1.52±0.37	$t = 2.36$
115 10 mg i. v.	10	2.87±0.59	$5\% > p > 2\%$	1.96±0.45	$5\% > p > 2\%$

The results obtained were similar in the rabbit experiments. No difference was detected between the two groups of rabbits 74 days after the injection of BCG and strain 115, respectively. No statistical analysis was done because of the small number of rabbits.

On analysing the results of our experiments on guinea-pigs, rats and rabbits, we came to the conclusion that no significant difference could be

observed between the residual virulence of the BCG Paris strain and that of strain 115 of Weissfeiler. This conclusion is well founded, because the methods used are suitable for studying the residual virulence of attenuated mycobacteria. The intraperitoneal injection of 5—10 mg of bacteria was recommended for this purpose by Rosenthal. According to his observations, 3—6 weeks after the injection marked lesions develop in the mesenteric lymph-nodes and these show the degree of the residual virulence of the strains under investigation. The study of the residual virulence by the use of mice injected intravenously was first recommended by Suter and Dubos. In the experiments reported here, white rats were used instead of mice. According to our experience over the last ten years we can state that white rats are very suitable for the study of such problems, moreover the rare occurrence of intercurrent diseases represents a further advantage. The residual virulence of BCG strains on rabbits was studied by Coulaud and Lurie and in Hungary by Weissfeiler, Karassova, Földes, Vincze and Gyenes. According to Coulaud large doses of BCG caused focal changes in the lungs, liver and spleen, but these lesions underwent regression later. In contrast, the Hungarian authors mentioned above observed a lethal effect as a result of the same doses. This difference can be explained by the different age of the cultures used by these authors, but it is certain that intravenous injection of large doses of mycobacteria with an attenuated virulence is a very suitable method for the study of the residual virulence of different strains.

The results obtained correspond with those of Šula, Šulova and Spurná, who found similar residual virulence of BCG and strain 115. in their experiments on guinea-pigs and rabbits. However, strain 115 proved to be more virulent in experiments on mice. The same authors found that INH resistant strain 115 has the same immunogenic effect as that of INH resistant BCG strains.

Summary

The residual virulence of the BCG Paris and the strain 115 of Weissfeiler was studied by intraperitoneal injection of guinea-pigs and intravenous injection of rats and rabbits. It was found that after administration of 10 mg of these bacteria, no significant difference could be established between the average body weights, spleen and lung weights of the animals injected with the two strains. Pathological and histological examination showed no difference between the two groups of animals injected with BCG and strain 115, respectively.

According to the experimental results, strain 115 of Weissfeiler can be recommended for controlled experimental immunization of human beings.

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VIRULENCE OF THE SENSITIVE MYCOBACTERIAL STRAIN 115 OF WEISSFEILER*

by

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In two previous reports (Šula et al. 1963a, b) entitled virulence and immunization effect of INH-resistant mycobacterial strain No. 115 "Weissfeiler" we have submitted the virulence and immunogenity of INH-resistant and PAS-resistant strain 115 of Weissfeiler to a detailed study. In this communication we report on our experience obtained in the study of the virulence of the sensitive variant of this strain.

On 13 February 1963, a 100 mg suspension was prepared from surface pellicle propagated for 3 weeks on Sauton medium with asparagine commencing on 31 January 1963. From the suspension viable counts were established on Jensen medium containing INH, STM and PAS, and on control Jensen and agar media in concentration of 10^{-6} mg (semidry weight of the strain). Contrary to the resistant variant, the strain was significantly inhibited even at a concentration of 0.1 gamma (for INH and STM) and 1 gamma (for PAS); see Table I.

Table I

Titration of INH—STM—PAS sensitivity

Petri dishes		1	2	3	4	5	6	7	8	9	10	Average
Control medium	Jensen	neg.	neg.	neg.	1.	neg.	neg.	neg.	neg.	neg.	neg.	0.1
	Agar	12	3	11	19	12	15	25	14	19	15	14.5
INH	0.1	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	—
STM	0.1	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	—
	1.0	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	—
PAS	0.1	21	18	12	14	16	8	14	9	10	15	13.7
	1.0	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	neg.	—

* Presented at the Scientific Conference of the Microbiological Research Group of the Hungarian Academy of Sciences, Budapest, October 1965.

TABLE II

Virulence (lung indices) of Myco

No. of rabbits	Dose	Weight		Weight decrease
		beginning	end	
228/63	10 mg	2.50	2.30	—0.20
227/63	20 mg	2.65	2.07	—0.59
11/63	25 mg	2.65	1.98	—0.67
230/63	25 mg	2.90	2.55	—0.35
9/63	25 mg	2.60	2.08	—0.52
229/63	50 mg	2.25	2.10	—0.15

On the whole the strain displays a better growth on agar that on Jensen media. In comparison with the same INH-resistant and PAS-resistant strain showing a markedly dysgonic growth on L—J media, the growth of the sensitive strain is more abundant, even when preserving a marked eugonic character of growth (Figs 1 and 2.).

Virulence Tests

The suspension was injected into 6 rabbits on 14 February and 28 March 1963 in the dose of 10, 20, 25 and 50 mg intravenously for each rabbit; 1 ml of the suspension containing 14,500,000 viable germs.

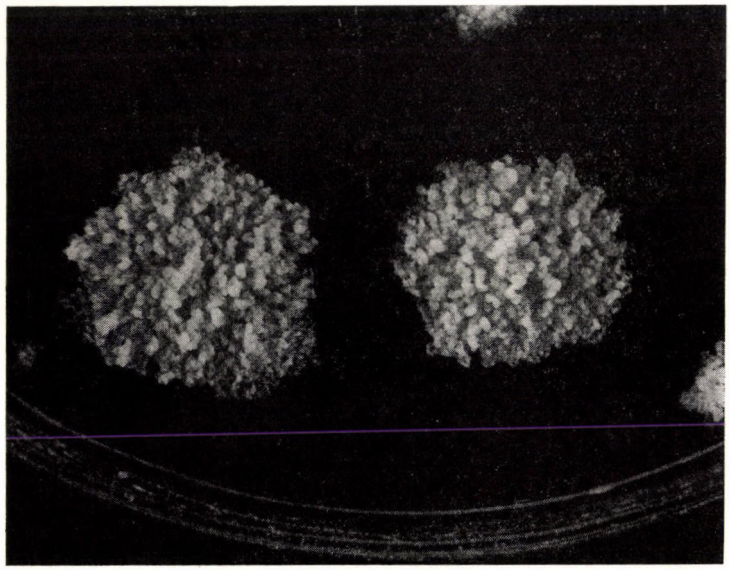

FIG. 1. Colony morphology W-sensitive strain 115. Agar medium. Age of culture 2 months

spec. 115 in rabbits (10 mg i.v.)

Lung	Liver	Spleen	Lung index
+++30.79	neg. 67.31	neg. 1.64	13.38
++56.39	neg. 123.42	neg. 3.18	27.24
75.21	+ 130.0	incr. 5.11	37.98
++++50.63	neg. 73.82	neg. 4.98	19.85
+++77.0	neg. 135.0	+ 6.45	37.01
+++23.07	neg. 61.62	neg. 1.99	10.98

The rabbits were observed for 2 months and then killed. Two of them died spontaneously, one after 12, and the second after 18 days. The lungs were removed, the findings were assessed macroscopically, and the weight of the lung was established in comparison with the total weight of the animal at the end of the experiment; in this way the lung index was determined. The results of these experiments are shown in Table II.

In the histological examination of the lungs, in all animals extensive, mainly caseous lesions were found afflicting the whole lung tissue extensively in such a way that the tuberculous process was of the same character as in pulmonary tuberculosis evoked in rabbits by *Mycobacterium bovis* (Figs

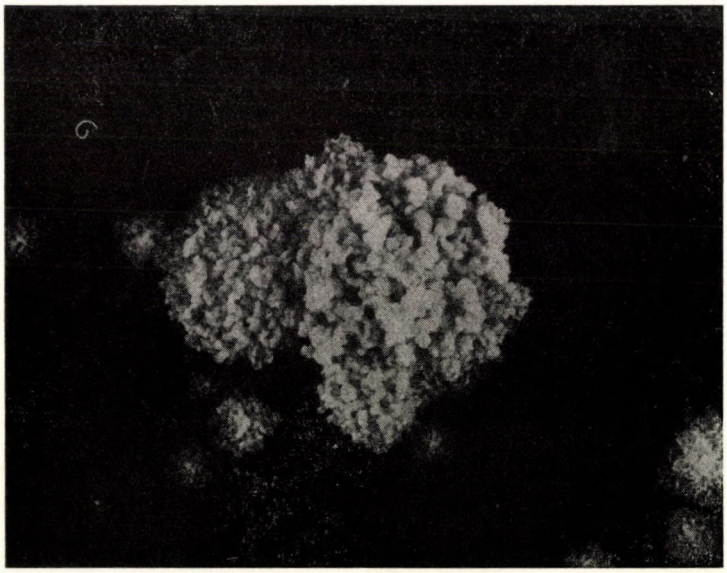

FIG. 2. Colony morphology W-sensitive strain 115. Agar medium. Age of culture 2 months

3 and 4). Simultaneously, in all rabbits injected with W—115 strains loss of weight was observed, which is a specific feature of a progressive tuberculous infection caused by *Myco bovis*.

FIG. 3. Lung pathology (20—25 mg i.v.) W-sensitive strain 115

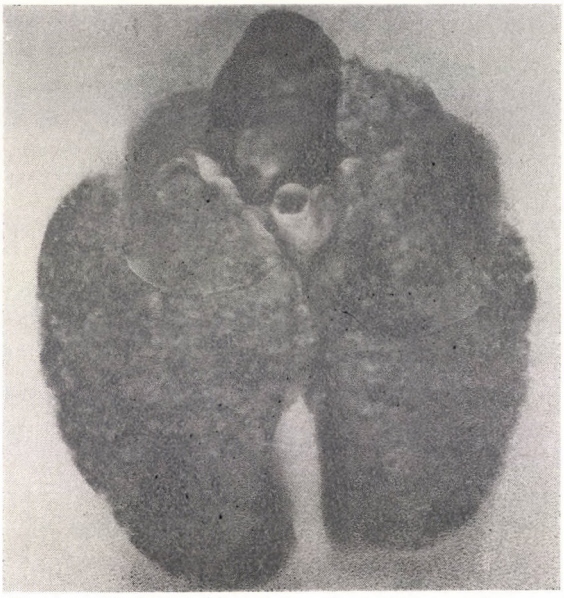

FIG. 4. Lung pathology (20—25 mg i.v.) W-sensitive strain 115

Together with the experiments in rabbits, guinea-pigs were injected with the same suspension in the dose of 10—100 mg, in groups of 10 animals each. After 2 months the guinea-pigs were sacrificed and investigated. The results are given in the Tables 3 and 4 and in Figs 5—8.

In the spleen indices, no substantial differences were observed when 10 or 100 mg of semidry weight of the Weissfeiler 115 strain was injected; these indices ranged between 0.40 and 0.53. Contrary to this, in omental indices,

TABLE III

Virulence of (Spleen indices) of Myco spec. 115 in guinea-pigs (100 mg i.p.)

Guinea-pigs	Weight		Increase ±	Spleen	Spleen index	Omentum	Omentum index
	beginning	end					
201	320	445	+125	1.06	0.23	8.21	1.84
202	310	390	+ 80	0.82	0.21	9.93	2.54
203	320	390	+ 70	1.35	0.34	18.06	4.63
204	320	450	+130	2.33	0.51	11.23	2.49
205	310	330	+ 20	3.34	1.01	6.66	2.01
206	310	320	+ 10	3.99	1.24	13.08	4.08
207	300	340	+ 40	1.13	0.33	4.26	1.25
208	300	290	— 10	2.53	0.87	3.97	1.36
209	300	430	+130	1.11	0.25	10.37	2.41
210	240	250	+ 10	1.02	0.40	1.87	0.74
Average	303	363	+ 60	1.86	0.53	8.76	2.33

TABLE IV

Virulence (spleen indices) of Myco spec. 115 in guinea-pigs (10 mg i.p.)

Guinea-pigs	Weight		Weight increase	Spleen	Spleen index	Omentum	Omentum index
	beginning	end					
91	320	400	+ 80	1.02	0.25	3.57	0.89
92	320	550	+230	2.01	0.36	4.76	0.86
93	310	300	— 10	2.43	0.81	3.04	1.01
94	300	410	+110	1.16	0.28	4.66	1.13
95	310	470	+160	1.23	0.26	4.68	0.99
96	310	400	+ 90	1.42	0.35	7.51	1.87
97	310	420	+110	3.28	0.78	3.63	0.86
98	310	370	+ 60	1.44	0.38	3.14	0.84
99	290	440	+150	1.47	0.33	10.57	2.40
100	300	500	+200	1.28	0.25	4.25	0.85
Average	308	426	+118	1.67	0.40	4.98	1.17

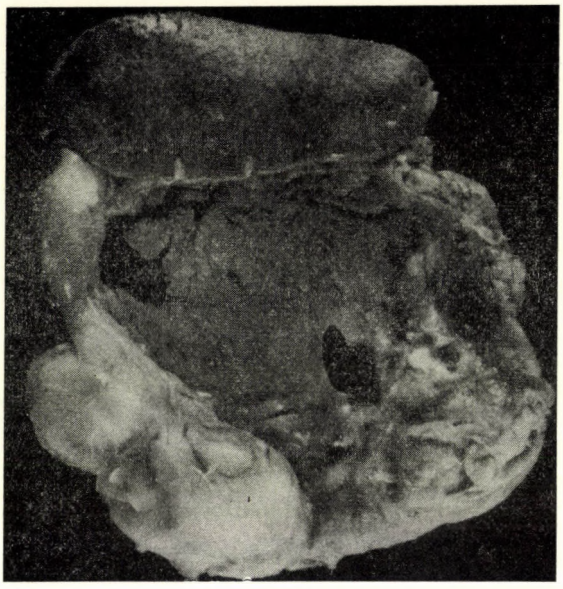

FIG. 5. Spleen and omental lymph-nodes of guinea-pigs injected intraperitoneally with 10—100 mg of W-sensitive strain 115

FIG. 6. Spleen and omental lymph-nodes of guinea-pigs injected intraperitoneally with 10—100 mg of W-sensitive strain 115

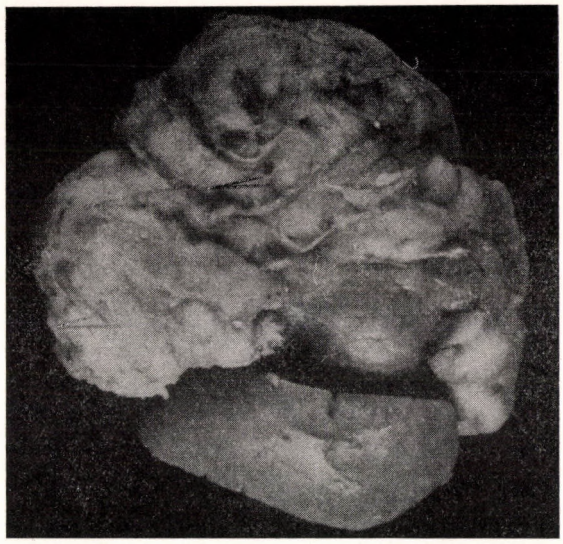

FIG. 7. Spleen and omental lymph-nodes of guinea-pigs injected intraperitoneally with 10—100 mg of W-sensitive strain 115

FIG. 8. Spleen and omental lymph-nodes of guinea-pigs injected intraperitoneally with 10—100 mg of W-sensitive strain 115

representing the proportion of the weight of the omentum and omental lymph-nodes to the weight of the whole animal at the end of the experiment, significant differences were established, corresponding to the dose of infection. When 10 mg was injected intraperitoneally, the average value of the omental index was 1.17, and when 100 mg was administered, the omental index was twice as high, i.e. 2.33.

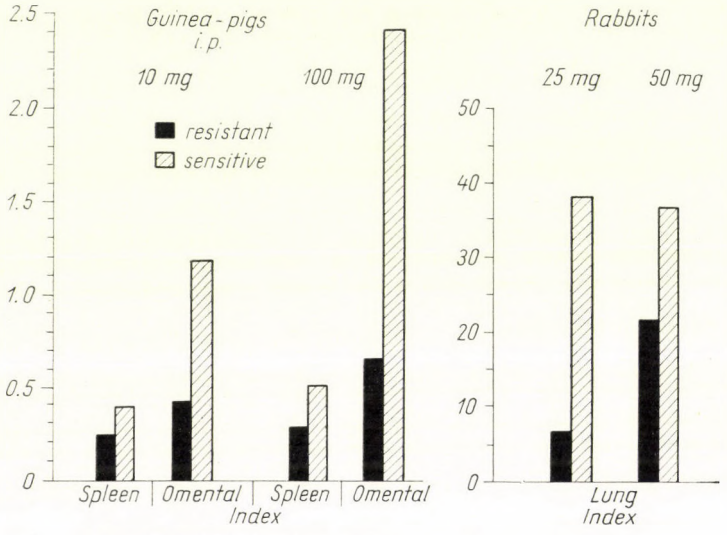

FIG. 9. Histogram. Virulence tests of Myco spec. Weissfeiler strain 115 sensitive and resistant to major drugs (INH and PAS)

When comparing these virulence values with the findings ascertained in the virulence study of the INH-resistant variant of the strain 115 of Weissfeiler, as shown in Fig. 9, we observed that the sensitive strain is more than twice as virulent as its INH-resistant and PAS resistant variant. In addition, in the histological examination of the liver and lung we found tuberculous lesions mostly in the form of milliary nods; this phenomenon is not observed in vaccine strains used in Czechoslovakia. For all these reasons we did not undertake any immunization experiments with the sensitive strain 115 of Weissfeiler, as we came to the conclusion that the strain is too virulent to warrant practical application for immunization in man.

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EXPERIMENTAL STUDY OF THE VACCINAL PROCESS INDUCED BY STRAIN NO. 115

by

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ATTEMPTS to develop more effective antituberculosis vaccines by improving vaccine strains are proceeding in two ways.

Some investigators study different BCG substrains which have been obtained by culturing BCG under variable conditions. The substrains are different from one another in numerous basic properties. Although such studies have been carried out for many years, it is still impossible to tell which of the substrains is preferable. The failure is due to the lack of a generally accepted experimental methods and of a method of assessment.

Other researchers are trying to improve the vaccinal effect by searching for new vaccine strains. At present only few works represent this direction. Among these, the work of Weissfeiler et al. concerning strain 115 deserves special attention These authors having studied the strain for years made it available for other laboratories including the Tarasevich Institute for the Control of Medical Biological Preparations where we have begun to study the strain.

Parallel with these studies the R_1Ra strain, which had been subjected to limited studies in small groups of humans in the USA, and the BCG strain were also investigated.

The biological activities of the strains were characterized by immunological, immunomorphological and allergic indices as well as by the residual virulence of the strains and their capacity to multiply in animals.

To study the allergizing capacities and the resulting immunomorphological changes simultaneously, experiments were carried out with 57 guinea-pigs distributed in three groups. These were given the R_1Ra strain which is an avirulent variant of *M. tuberculosis* (group I), Weissfeiler's strain 115 (group II) and BCG (group III), respectively.

The vaccines were administered intradermally at the inguinal region in two doses, viz. 0.05 mg on the left and 0.02 mg on the right side. Tuberculin test was carried out before vaccination, and on 15 occasions between the third and 155th postvaccination days.

Samples for morphological investigation were taken from visceral organs and lymph-nodes from one guinea-pig of each group on every occasion when tuberculin test was carried out. The data reflecting allergization of the experimental animals are shown in Fig. 1. It is seen that in general positive allergic reaction (5 mm or more in diameter) was first observed between the 2nd and 7th days after injecting 100 TU PPD. The guinea-pigs inoculated with strain 115 developed positive tuberculin reaction somewhat sooner,

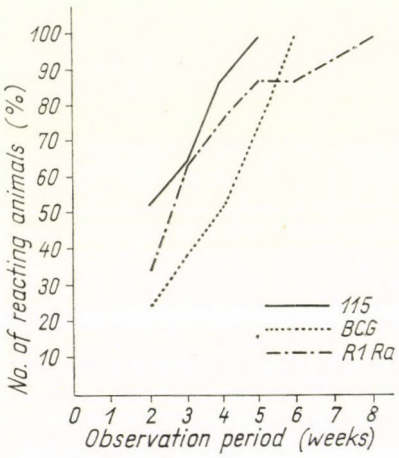

FIG. 1. Number of animals showing postitive reaction to 100 TU PPD at different times after vaccination

and the reaction of these animals was somewhat stronger. By the end of the second week 53, 35 and 24% of the animals vaccinated with strain 115, R_1Ra and BCG, respectively, had become positive. All the animals in the respective groups became positive by the end of the 5th, 8th and 6th postvaccination week.

The general results of the morphological studies are as follows. Each of the strains induced standard immunomorphological reactions in the organs possessing reticular tissues (lymph-nodes, spleen) and in the lungs. These reactions were characterized by the production of non-specific globulins and specific antibodies. They manifested in a general hyperplasia of lymph-node cells. The hyperplasia extended to all kinds of lymph-node cells, but the enlargement of the germinative centres with numerous mitotic formations and the proliferation of the sinus cells showing the desquamative catarrh phenomenon were especially pronounced. Immunogenesis was indicated by plasmacytogenesis, i. e. appearance of plasma blasts of different degrees of maturity. The degree of plasmacytogenesis was considered as the

measure of immunogenesis. Besides these processes, development of specific epitheloid granulomas was characteristic of the mycobacterial antigen stimulus. The granulomas might be attributed to the residual virulence of the respective strain.

Though there were some common features in the immunomorphology of the strains under study, each strain showed some particularity as to the rate, course and intensity of morphological reactions. The earliest and most intensive lymph-node reaction was caused by strain 115. This reaction was observable even on the 5th day, thus, preceding the reaction due to the R_1Ra and BCG strains by two days. Consistently with the general observations that there is a "latent period" in every antibody production, tuberculin sensitivity was always preceded by the morphological reaction. Subsequently, from the 9th day on, active reaction characterized by plasmablasts and immature plasma cells was observed in each group; the reaction affected in groups I and II all the lymph-nodes examined, and in group II (strain 115) only some of them. During the subsequent 60 days, plasmacytogenesis was of about the same intensity in each of the groups; in the same period fibrosis began in groups I and III and the process tended to decline; at the same time in group II the activity remained unchanged. In this group plasmacytogenesis in the lymph-nodes was still active on the 96th day, whereas in groups I and III plasmacytogenesis flared up for a short time after the period of rest and atrophic alterations. In the guinea-pigs of group II this activity was continuous during the whole period of observation (142—153 days) while it stopped in groups I and II; it was less pronounced in group I (R_1Ra strain) than in group III (BCG).

Epitheloid-cell granulomas of specific structure were observable only in groups II and III. It is of interest that their incidence corresponded to the biological activity of the respective strain. In the group immunized with strain 115, 5 out of the 19 animals showed specific alterations. These even appeared relatively early (on the 23rd, 32nd, 112th, 121st and 142nd days). Initially, the regional and the tracheo-bronchial lymph-nodes were impaired; it was as late as on the 121st day that lymph-nodes of other regions became involved. In the lymph- nodes of BCG-inoculated animals, granulomas were found in 3 out of the 19; only one regional lymph-node was impaired in each of these cases, at a relatively late time (62nd, 142nd and 153rd days). These specific formations had a structure characteristic of vaccinal granulomas; no collagenization was observed. The appearance of specific structures in group II (strain 115) was due to an active non-specific immunological reaction, whereas in group III (BCG) it resulted from some decreased activity.

To determine the residual virulence of the strains under study, guinea-pigs were inoculated intraperitoneally by Nakhimson's modified method. Each culture (115, R_1Ra and BCG) was administered intraperitoneally in doses of 10 mg. Control animals were given 0.5 ml saline. A total of 120 animals were used in these experiments. Forteen days after introduction of the culture, the guinea-pigs were killed, the omentums were weighed and

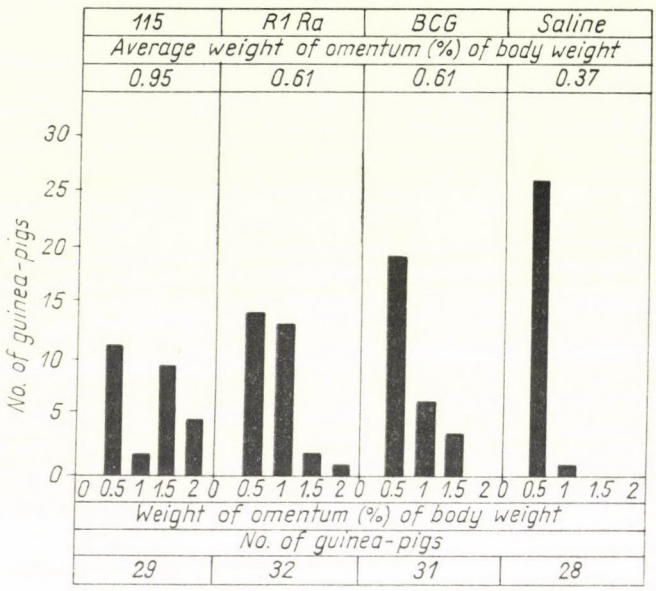

FIG. 2. Weight distribution of omentums of guinea-pigs

the omentum's weight in percentage of the body weight was calculated. The results are shown in Fig. 2 and the Table below. It is clear that strain 115 had the greatest residual virulence, while that of the strains R_1Ra and BCG was about the same. As regards the impairment in the omentum the three strains significantly differed from one another.

The residual virulence of strains 115 and BCG was compared in chick embryos, too.

For this purpose eight-day-old chick embryos were used. The egg shell was bored at the centre of the air sac and at the side of the egg between the large veins. The eggs so prepared were laid with their bored side upwards. Then the egg membrane over the air sac was opened with a sterile surgical needle. On to the other opening about 0.1 ml of MacIlvain buffer (0.004 M) was dripped (the buffer was pre-heated to 50° C); then the egg membrane was opened by pressure. Thus, one drop of buffer, getting under the egg

membrane separated a part of the chorio-allantoic membrane from the egg membrane. The separation became complete when air was sucked off through the hole over the air sac.

Average weight of the omentum (expressed in per cent of body weight) 14 days after intraperitoneal injection of the indicated attenuated strain of *M. tuberculosis*

Strain	No. of animals	Average weight of the omentum in percentage of body weight	
		M ± m	±
115	29	0.95+0.06	+0.3
B+G	31	0.61+0.05	0.26
RlRa	32	0.61+0.04	0.24
Control	28	0.37+0.008	0.05

Before introducing bacteria, the embryos were placed in an incubator of 38° C for at least 2 hours. Subsequently, inoculum was introduced into the lateral opening of the egg shell. Finally the holes were covered with adhesive tape.

The infected embryos were re-incubated for 10 days, and then the chorio-allantoic membranes were examined.

In this series of experiment 39 embryos were inoculated with BCG and 38 embryos with strain 115; 0.5 mg bacterium was inoculated in 0.1 ml. The results are shown in Fig. 3.

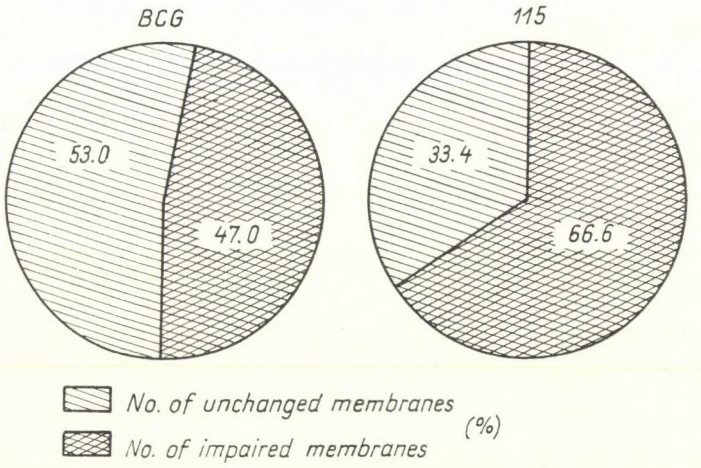

FIG. 3. Frequency of impairment of the chorio-allantoic membrane after introduction of BCG or strain 115

It is clearly visible that the residual virulence of strain 115 exceeds that of BCG (66.6 and 47.0% of the membranes were found to be impaired).

Obviously, the experiments carried out in animals and in chick embryos have unequivocally shown that the residual virulence of strain 115 is higher than that of BCG.

Another characteristic of the vaccine strains, namely, the capacity of implantation in organs and lymph-nodes of experimental animals was

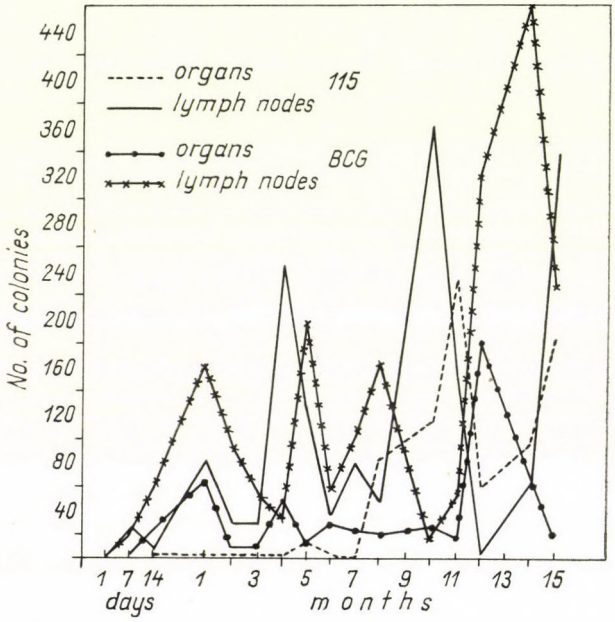

FIG. 4. Implantation of BCG and strain 115 in organs and lymph-nodes of white mice

examined in three groups of white mice, 291 mice altogether. Each mouse was given 0.1 mg of one of the strains under study. After 24 hours, 1 week, 2 weeks and 1, 2, 3, 4, 5, 6 and 7 months, 5 mice from each group were killed and the lymph-nodes of the killed mice was inoculated into Gelberg's medium. Colonies grew from the lymph-nodes of the mice inoculated with the R_1 strain only in the early phase of infection, whereas in the case of strains 115 and BCG growth was undulant (Fig. 4). For strain 115, the cultivability index fluctuated between 0.2 and 245, for BCG between 2 and 196, colonies during the period of observation. The number of colonies cultivable from the lymph-nodes was the highest on the 120th day (245 colonies) and 150th day (196 colonies) in the case of strain 115 and BCG, respectively; subsequently, the number of colonies decreased until the

180th day when 37 and 58 colonies were counted. Thereafter another increase ensued up to 80 colonies (strain 115) and 102 col onies (BCG). The average number of colonies obtained from the organs fluctuated between 1 and 14 colonies and 1 and 66.4 colonies for strain 115 and BCG, respectively. Strain 115 reached the highest number of colonies (14) on the 150th day, whereas BCG (66 colonies) on the 30th day. Subsequently, this index declined till the 210th day when strain 115 could no longer be recovered, while BCG provided 24 colonies per mouse. It has been concluded that strain 115 can be implanted to about the same extent as BCG.

The immunogenicity of the three strains was compared in an experiment with 216 guinea pigs. These were distributed in four groups. Groups I—IV were given strains 115, R_1Ra, BCG and saline (control), respectively (Fig. 5).

For vaccination purposes, 12-day Sauton cultures (2nd generation) were used. The inoculum (0.01 mg in 0.5 ml) was injected subcutaneously into the right inguinal region. The animals in all four groups were challenged with 0.0001 mg (in 0.5 ml) of the bovine strain Ravenel. From each group 30 guinea-pigs were killed on the 115th day after infection. By that time in the control group 2 animals had already died of generalized tuberculosis. The survival time of the other animals was observed.

The following results were obtained. Nakhimson's tuberculosis index was 68.0 for the control group, 68.5 for the group immunized with the R_1Ra strain, suggesting that postvaccinal immunity cannot be brought about with the R_1Ra strain. The same index was 36.0 and 31.0 for strain 115 and BCG, respectively. The difference between the last two values is not significant statistically, showing that these two strains are about equally active immunologically. The survival times are consistent with these conclusions. The average survival time in days was 218 for both groups I (115) and III (BCG), whereas 166 and 173 for groups II (R_1Ra) and IV (control), respectively.

Conclusions

A comparison of the biological activities of the Mycobacterium strains 115 (Weissfeiler), BCG and R_1Ra has led to the following conclusions.

1. Strain 115 possesses the strongest, R_1Ra the weakest, allergizing capacity.

2. As regards the basic immunomorphological properties, all three strains are equivalent; but in the case of strain 115 the reaction in the regional and promote lymph-nodes, in the lungs and in the spleen appears sooner and is more intensive.

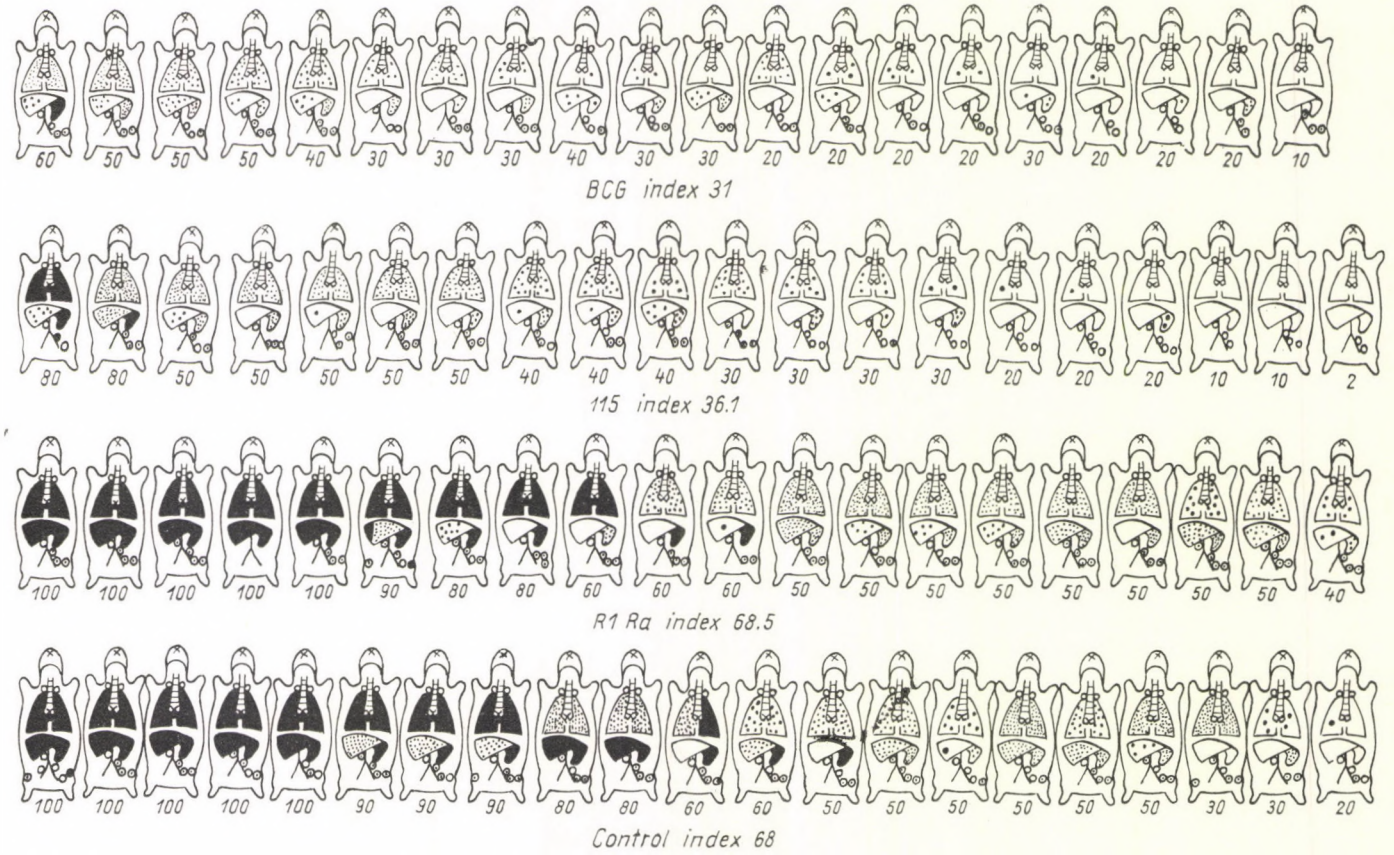

FIG. 5. Comparative assessment of the immunogenicity of BCG, strain 115 and Rl Ra 115 days after challenge with a virulent strain

3. The development of specific formations — epitheloid cells surrounding tubercles — was not regular because of the little inocula. Nevertheless, the earliest and most frequent appearance of these phenomena was observed in the animals inoculated with strain 115, suggesting that the residual virulence of this strain is higher.

4. Strain 115 showed the highest residual virulence even on the basis of the impairment of the omentum (both in intraperitoneally inoculated guinea-pigs and in chick embryo experiments). This index was about the same value for the strains BCG and R_1Ra.

5. As to the implantation and multiplication in the organs of experimental animals, strain 115 is not different from BCG.

6. The immunogenecity in guinea-pigs of strain 115 proved to be approximately identical with that of BCG.

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HISTOLOGICAL LESIONS INDUCED BY STRAIN 115 IN GUINEA-PIGS, ALBINO MICE AND CHICK EMBRYOS AND THE IMMUNOGENIC PROPERTIES OF THE STRAIN

by

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THE IMPORTANCE of specific prophylaxis, besides social and public health measures, is well known. The protective effect of BCG vaccine against severe and acute forms of tuberculosis is obvious. However, the immunity acquired by immunization is of relatively short duration and not sufficiently. For this reason, attempts have been made to develop new vaccination procedures (A. De Assis) and new vaccines (Weissfeiler, Šula, Wells).

Weissfeiler, by his own adsorption method (regeneration of filtrable forms), recovered an avirulent *Mycobacterium tuberculosis* typus humanus strain from the blood of a patient suffering from fibrocavernous tuberculosis. The strain has been designated attenuated tuberculosis strain No. 115 (in the following, strain 115).

According to the investigations of Weissfeiler et al. (1940—1965), the avirulence of the attenuated strain is persistent and hereditary. It does not change even in the course of passages in susceptible animals. The strain survives in the animals for long periods of time, it preserves its viability without causing any progressive tuberculous process. Only hyperplastic-proliferative processes and, to a limited extent, proliferation of epitheloid cells develop which, however, completely regress subsequently. The anti-tuberculous immunity due to strain 115 is, according to the authors, stronger than that supplied by the BCG vaccine.

We received the strain 115 on Löwenstein medium from Prof. Weissfeiler in June, 1959. We cultured it on Sauton and Löwenstein media and in glycerol-potato; transfers were made at intervals of 14—15 days. In the course of parallel cultivation of the strain on the three different media, we were unable to notice any change in its morphology as compared with the morphological features seen in the first subculture made in this laboratory.

In several species of experimental animals we observed the tissue lesions caused by different doses of strain 115 when the bacterium was injected by different routes. The reactions were compared with those brought about by the same doses of BCG. The experiments were carried out in 184 guinea-

TABLE I

Dilution of tuberculin	Observation													
	day							month						
	5	10	15	20	30	40	50	2	3	4	6	9	12	18
1 : 10	—	5	6	7	10	12	14	17	17	15	14	10	6	4
1 : 100	—	3	3	4	4	5	5	7	10	12	9	6	4	—
1 : 1000	—	—	—	—	—	—	—	5	5	—	—	—	—	—
1 : 10000	—	—	—	—	—	—	—	4	3	—	—	—	—	—
Non-specific lesions	+	++	++	+++	+++	+++	+++	++	+++	++	+	—	—	—
Specific lesions	—	+	++	++	+++	+++	+++	++	++	+	—	—	—	—

Note: + = lesions at the site of injection and in the regional lymph-nodes
++ = lesions extend to the spleen and remote lymph-nodes
+++ = lesions extend to the lungs and the liver; the data represent average indices.

pigs, 110 albino mice, 8 rabbits, 186 chick embryos and 8 snakes (*Vipera ammodytes ammodytes*). Some of the experiments carried out in 1959 were performed in collaboration with Dr. Sr. Rodopska and Dr. G. Tenchev. The animals were inoculated with 14-day-old Sauton culture in saline. For histological and histochemical purposes, the material was prepared by some special methods, in addition to the usual techniques.

Parallel with the morphological examination of the lesions, the dynamics of the tuberculin reaction was observed in a group consisting of 40 guinea-pigs (Table I). The cutaneous tuberculin reaction was tested with Alt-tuberculin (in 1 : 10, 1 : 100, 1 : 1000 and 1 : 10,000 dilutions). Tuberculin was injected intradermally at 4 sites on the back. The sites of inoculation were at a distance of 4—5 cm from one another. The tuberculin reaction was read on the basis of the diameter of the infiltration after 48 hours.

The immunogenic properties of strain 115 were evaluated on three groups of guinea-pigs, each group consisting of 40 animals (Table II). Each animal was immunized with 0.1 mg bacterium suspension, and challenged with 0.0001 mg of the Ravenel strain of *M. tuberculosis* two months later. The changes that ensued in the biological condition of the animals were registered by consistent observation. The general condition, body weight, local reactions and pathomorphological lesions were registered. The degree of tuberculous injuries were scored and expressed by an index (tuberculosis-impairment index). According to the scheme applied the value of this index was the highest in the control group. This value was 27.

TABLE II

Organs		Control group only Ravenel	Experimental group BCG + Ravenel	Experimental group 115 + Ravenel
Lymph-nodes	weight	3	2	3
	index	6	4	4
Spleen	weight	6.4	2.8	2.4
	index	9	6	3
Liver	weight	40	33	32
Lungs		8	4	4
General index		27	16	14

Note: + mild lesions ++ medium lesions +++ strong lesions

+ is scored in relation to lymph-nodes 1
„ „ the spleen 2
„ „ the liver 3
„ „ the lungs 4

As to the development of morphological changes in the guinea-pigs, most of the lesions were localized at the site of injection of the bacterial suspension, in the regional and remote lymph nodes, in the spleen, lungs, liver and, in some cases, in other organs, too. Most of the lesions were of two types: 1. non-specific, hyperplastic, proliferative processes extending to the whole reticulocyte-histiocyte system; 2. specific lesions manifesting in formation of

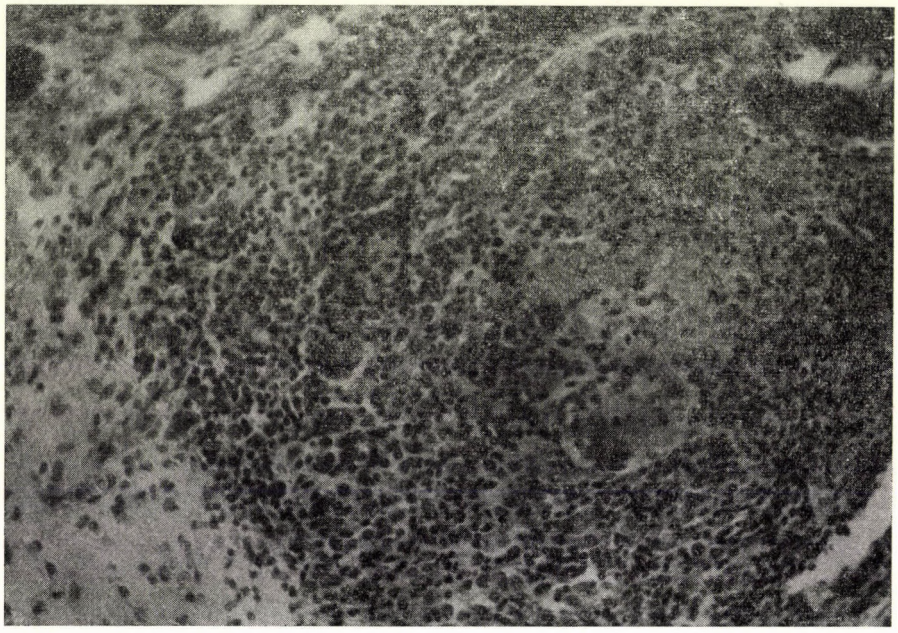

FIG. 1. Inflammatory focus at the site of introduction of 0.1 mg of strain 115; 20 days after infection. Haematoxylin-eosin stain; × 120

limited proliferations of epitheloid cells around the site of injection, in the regional lymph-nodes and, occasionally, in the spleen.

At the *site of injection* (subcutaneous inoculation) non-specific inflammation developed in the connective tissue even on the first or second day. The focal inflammation mainly contained leucocytes and histiocytes with acid-fast bacteria among cells. In the period from 5 to 10 days after inoculation, dystrophic processes developed in some of the cells in the infiltrated area (Fig. 1) and a thin capsule of connective tissue was formed around the inflammatory foci. The larger the inoculum the more pronounced was the dystrophic process. On comparison with the inflammatory processes due to BCG, these exudative and necrobiotic processes were more pronounced and developed more rapidly. Some of the macrophages in the inflammatory

foci underwent a transformation to form a granuloma of epithelioid cells with central necrosis by the 15th—20th day. Among the epithelioid cells, Langhans type cells 60—70μ in diameter appeared, each possessing 8—10 monopolar-arranged nuclei. By the 30th—40th day in the granulomas a large necrotic focus had developed which was surrounded by a narrow streak of epithelioid cells and fibroblasts. By the 60th day absorption phenomena were observed in the tubercle, and 90—120 days after inoculation a small fibrous granule remained at the site of the former lesion. Persistent abscesses were often observed. After intraperitoneal injection, as well as after injection of BCG, fibrinous exudate and aggregation of leucocytes appeared on the serosa of the abdominal organs, especially in the omentum. The serosa of the omentum was thickened along the greater curvature of the stomach. Histological examinations have shown that the thickening of the omentum was due to an infiltration of the serosa with leucocytes, histiocytes, lymphocytes and a proliferation of the perivascular mesenchyma. Besides, cellular transformation of histiocytes into epithelioid cells was observed. As compared with the morphological picture following BCG vaccination, the inflammatory process developed faster, exudative and necrobiotic processes were predominant and the proliferative ones were less pronounced. Consequently, by the 15th—20th day well-developed microabscesses were observable. These grew gradually and became visible to the naked eye 20—30 days after inoculation. A great number of acid-fast bacteria were present in the abscesses. Since the exudative processes prevailed over the proliferative ones and abscesses developed earlier, the omentum did not thicken to such a degree as after BCG vaccination; it was macroscopically different and coalescence with the adjacent organs occurred more frequently. By the 40th—60th day resorptive symptoms appeared in the thickened omentum. These disappeared by the 90th—120th day. Synechiae between the omentum and adjacent abdominal organs persisted for a longer times. The fibrious adhesions, abscesses and synechiae were more persistent after inoculation with larger doses of strain 115.

In the *regional lymph-nodes*, inflammatory hyperaemia, proliferation of the reticuloendothel and hyperplasia of the lymphoid tissue were observable. In the period 10—15 days after inoculation, foci of epithelioid cells appeared in the intermediary zone (Fig. 2). In these foci necrobiotic changes developed dy the 20th—30th bay and a small necrotic focus was formed in each. When the necrotic focus had appeared, the hyperplastic processes were reduced. Consequently, only a mild hyperplasia of the lymph nodes was recognizable macroscopically. In remote lymph nodes, parallel with strengthening of the non-specific hyperplastic proliferative processes, ribonucleic

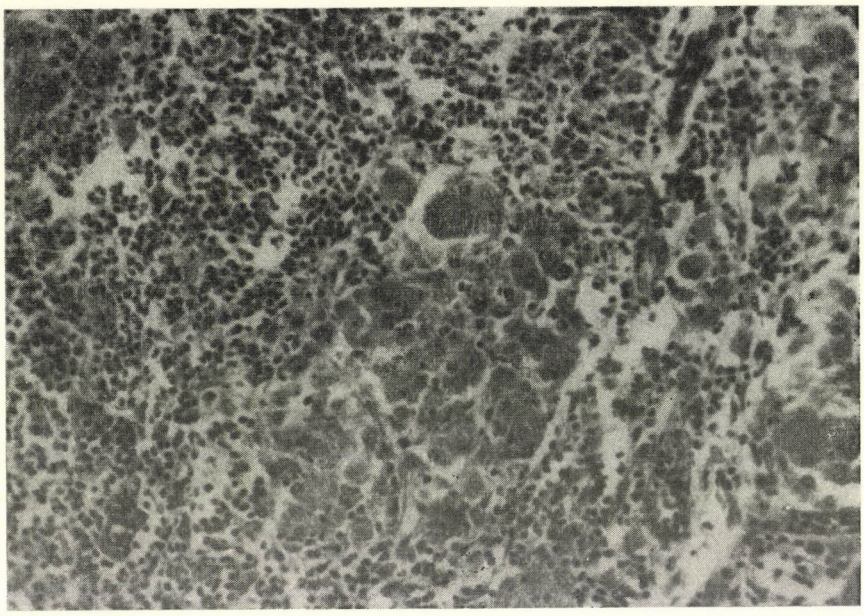

FIG. 2. Epithelioid-cell focus in the regional lymph-node 20 days after injection of 0.1 mg of strain 115. Haematoxylin-eosin stain; × 120

acid and glycogen increased in quantity and the alkaline phosphatase and peroxidase activities as well as the phagocytic activity of the macrophages also increased (Fig. 3). Resorptive symptoms were observed in the granulomas 40—60 days after inoculation. Subsequently they disappeared.

In the *spleen,* the non-specific reactive alterations extended first of all to the white pulp and manifested itself in a moderate hyperplasia of the malpighian bodies. This was the strongest between the 20th and 40th days, then gradually declined and disappeared. After intraperitoneal introduction of massive doses, small epithelioid-cell granulomas developed in the perifollicular zones of malphighian bodies (Fig. 4). Later these granulomas were absorbed. In the spleen, the necrobiotic processes were more pronounced and the fibrous foci that had been left behind after absorption of tubercles persisted longer than after BCG vaccination. Nevertheless, after subcutaneous injection, epithelioid-cell foci were very rarely formed.

In the *lungs*, lymphohistiocytic infiltrations formed after 20—30 days. These were similar in cell composition and development to the infiltrations due to the BCG strain. Histological examinations have shown that the former were smaller and consisted, first of all, of lymphocytes, perivascular infiltrates and lymph-nodes. It is well known that lympho histiocytic in-

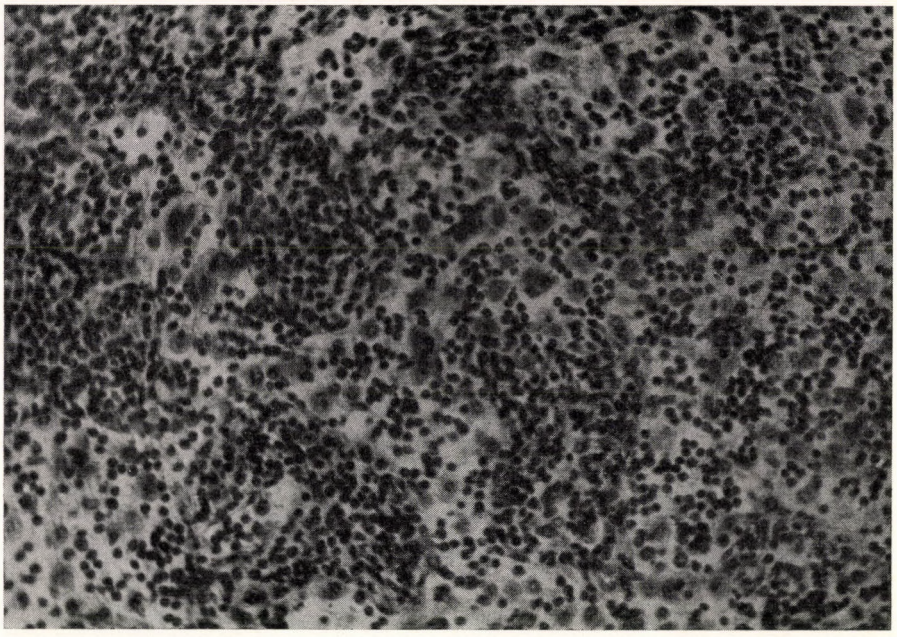

FIG. 3. Strong macrophage reaction in the paralumbal lymph-node. Brachet stain; × 240

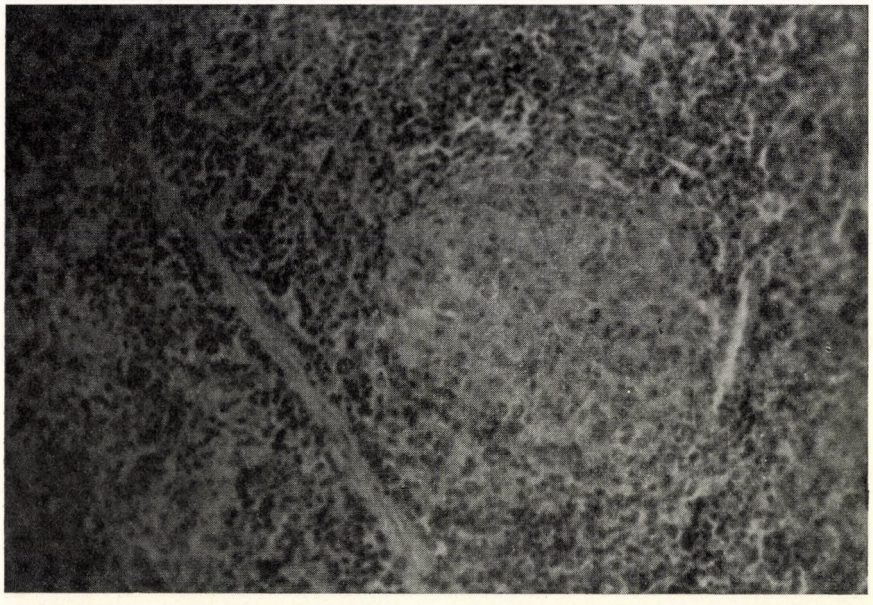

FIG. 4. Epithelioid-cell focus in the spleen. Haematoxylin-eosin stain; × 120

filtrations are considered to be characteristic of general immunomorphology (Ja. L. Rapoport 1957, 1963; D. P. Stoyanov 1962). In some cases, besides non-specific processes, pneumonic foci appeared without epithelioid cells and acid-fast bacteria in them. Foci of epithelioid cells failed to be observable even after introduction of large doses.

In the *liver*, non-specific reactive lesions and proliferation of epithelioid cells developed only after intraperitoneal injection of the strain. The

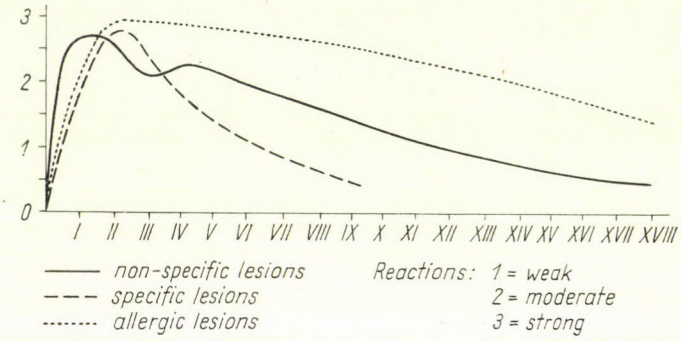

FIG. 5.

cellular granulomas may attain 70—80 μ in diameter. By the 40th—60th day the granulomas were absorbed and disappeared.

In other organs, non-specific lesions resembling those seen after BCG vaccination developed (Fig. 5).

According to the observations of Weissfeiler et al., the histological lesions developing in the guinea-pigs as a result of infection with strain 115 are hyperplastic-proliferative and characterized by demarcated epithelioid proliferations. The epithelioid-cell granulomas developed primarily at the site of injection and in the regional lymph-nodes; after intraperitoneal injection also in the omentum spleen and liver.

The size and extent of the proliferations depended on the size of the inoculum. These, like the epithelioid-cell proliferations due to BCG, always disappeared. The proliferations caused by the attenuated *M. tuberculosis* strain 115 are different histogenetically from those caused by BCG in some morphological characteristics, namely:

1. exudative and necrobiotic processes appear earlier and develop stronger;
2. the proliferative component of the inflammatory process is less pronounced;
3. a well-defined capsule of connective tissue is observable;

4. the hyperplasia of the lymphoid tissues is definitely suppressed during epithelioid proliferation.

On the basis of these morphological characteristics we believe that strain 115 is more pathogenic for the guinea-pig than BCG, but does not cause fatal infection. The epithelioid proliferations completely disappear in every case. There are no differences in the histochemical changes characteristic of an increased biological activity of the macrophages (as regards ribonucleic acid and glycogen content and alkaline phosphatase activity).

Numerous authors have investigated the correlation between tuberculin sensitivity and tuberculous lesions. Most authors suggest that the presence of tubercles in the macro-organism is a prerequisite of tuberculin allergy (Grüner and Hamburger 1910; Helmholz and Loyofuku 1910; Ebert, Ahern and Bloch 1948). The tuberculin sensitivity of BCG-inoculated animals was studied as a function of time by Boquet and Bretey (1934) and O. Drabkina (1959). According to V. I. Puzik (1956) and D. P. Stoyanov (1962), the conversion of tuberculin reaction after BCG vaccination coincides with the development of paraspecific reactions. Such a coincidence may be observed both during the vaccinal process and in the early phase of the tuberculous inflammation. According to Ya. L. Rapoport and L. I. Nahimson (1957), the increase in tuberculin sensitivity coincides with the first signs of the immunomorphological reaction, without the appearance of specific cells.

Correlation between tuberculin sensitivity and morphological changes in guinea-pig after vaccination with strain 115 does exist; the histological changes precede the development of tuberculin allergy. However, the latter is substantially more persistent than former.

As shown in Table I and Fig 1, there is some fluctuation in the development of both tuberculin sensitivity and histological changes; the development is relatively rapid during the first two months, it reaches its peak between the second and sixth months, then it declines gradually. The development and persistence of tuberculin allergy need a longer time than those of the morphological reactions.

On the first five days the tuberculin skin test is negative. Between the 10th and 15th days the animals either do not react or do so weakly to the first two dilutions of tuberculin. The skin reaction becomes positive by about the 20th day; it increases gradually and in the period from 40—60 days after inoculation the guinea-pigs begin to develop positive reaction with the third dilution of tuberculin. Tuberculin sensitivity reaches its highest value during the 3rd—6th months, when all animals give a positive reaction to the third dilution, some of them even to the fourth one. Subsequently, tuberculin sensitivity gradually declines first to the higher dilutions;

the animals remain sensitive to the larger concentrations for a longer time (in our experiment up to 18 months).

The evolution of morphological lesions approximately coincides with the appearance of tuberculin sensitivity. 1. Non-specific inflammation and isolated foci of epithelioid cells at the site of introduction of strain 115 (primary effect) as well as 2. hyperplastic-proliferative phenomena and foci of epithelioid cells in the regional lymph-node (primary complex) appear when the tuberculin test is still negative or weakly positive (on the 15th day).

Parallel with the appearance of positive tuberculin reaction between the 20th and 30th days after administration of the first or second dilution, the epithelioid-cell foci in the primary complex increase in size and well-defined non-specific hyperplastic-proliferative processes develop in the spleen and in remote lymph-nodes. At the appearance and during the maximum intensity of the tuberculin reaction (from the 2nd to the 6th month), the specific lesions extend to the whole primary effect, a great part of the tissues of the regional lymph-nodes, some parts of the spleen and, occasionally, to small foci in remote lymph-nodes (40th—60th day). Tuberculin sensitivity begins to decline parallel with the absorption of the epithelioid-cell formations and with the decline and cease the hyperplastic processes in lymph-nodes, in the spleen and in the intramural lymphoid tissues.

*

The experimental data concerning the immunogenic properties of strain 115 are shown in Table II. It is seen that, like BCG, strain 115 gives rise to an intensive antituberculous immunity, but the immunity induced by the latter strain is stronger. The resistance to tuberculosis manifests itself in morphological phenomena: viz. 1. in a lesser extent of necrobiotic and necrotic lesions, and 2. in the predominance of proliferative processes over the exudative ones. Nevertheless, long-term observation are still needed to decide whether the following generations of strain 115 will always result in a strong antituberculous immunity, and to what degree this immunity will exceed that resulting from BCG vaccination. It would be reasonable to carry out further similar experiments, for according to our observations obtained over many years, early generations of the BCG strain result in much stronger histological lesions than the later generations. However, when the BCG strain had been cultured in glycerol-potato medium containing bovine bile, the lesions became more intensive. A similarly reduced intensity of the morphological changes was observed in white mice inoculated with later generations of strain 115.

*

In white mice we found the morphological lesions localized first of all in lymph-nodes, the spleen, the liver and the lungs. In the lymph-nodes the reactive changes manifested in a hyperplasia of the lymphoid tissue and in formation of epithelioid-cell granulomas. In the regional lymph-nodes the hyperplastic processes reached their maximum development between the 20th and 30th days. Then hyperplasia declined and disappeared. In connection with the hyperplasia, small foci of epitheloid cells appeared and became visible to the naked eye 20—30 days after inoculation. Some of the epithelioid cells in the centre of the granuloma became pyknotic and dissolved. After the introduction of large doses, the dystrophic processes were more pronounced. In such cases a necrotic focus developed in the centre of the granuloma. This persisted for a short time and it was followed by absorption of occasional larger necrotic foci was slow. Such foci persisted in the regional lymph-nodes as miliary formations.

In the white pulp of the spleen pronounced hyperplasia developed; it became observable between the 30th and 40th days. As a result of hyperplasia, the spleen grew to 3—4-fold its original weight. Microscopically, in addition to the hyperplastic and proliferative processes, small foci of epithelioid cells were observable which were localized in the perifollicular zones of the malpighian bodies. Subsequently, the epithelioid proliferations were completely absorbed. After they had disappeared the hyperplasia of the white pulp gradually ceased. In the liver small lymphohistiocytic aggregations appeared between the 20th and 30th days. Later these disappeared. Foci of epithelioid cells with central necrosis developed after intraperitoneal injection of large doses. In the lungs infiltrative thickenings of some subpleural alveolar septa were observed.

The histogenesis of the changes and the dynamics of their development were about the same in the case of strain 115 as after inoculation of BCG. Nevertheless, we had the impression that in the tubercles formed in the regional lymph-nodes necrotic foci developed more frequently in mice infected with strain 115. These tubercles persisted for a long period of time (4 months). Moreover, in mice the first generations of the strain caused stronger lesions than the later ones.

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The general clinical condition of rabbits inoculated with strain 115 showed no change. The morphological lesions were most intensive in the spleen and in the lungs. In the spleen a moderate hyperplasia developed which became most prominent between the 20th and 40th days. This was

substantially more pronounced than in guinea-pigs and in mice. A similar species-specific reactivity is observable after the injection of BCG. At the time when the hyperplastic processes reach their greatest intensity, small epithelioid foci occur in the perifollicular zones of the malpighian bodies which, however, disappeared by the 60th day. In the lungs small pneumonic foci appeared between the 20th and 40th days. In some of these, small aggregations of epithelioid cells and fibroblasts were detectable. Subsequently, the epithelioid-cell proliferations regressed and disappeared.

According to the histogenesis and the dynamics, the morphological lesions in rabbits are similar to those caused by BCG. Profound and irreversible dystrophic lesions do not develop.

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In the membranes and organs of the chick embryo, the pathomorphological processes caused by different antigens show a great variability, e.g. disturbed blood and lymph circulation, various forms of inflammation and dystrophy, hyperplastic processes, etc. The reactive processes of the chorioallantoic membrane are phylological and ontological prototypes of the processes of a complex organism (Lang, Erich, and Cohn 1930; Brieger 1951, Rapoport 1958 etc.), their development is in no relation to the vasomotoric and trophic innervation. The lesions come about on the immediate stimulus of the introduced antigen. However, their evolution ensues very fast, within several days (Costil and Bloch 1938; Mur 1942; Rapoport 1958), in contrast to the mature organism, where it needs several weeks or months.

We kept under observation the development and histogenesis brought about by strain 115 in the membranes of the chick embryo. Only limited morphological lesions were observed with fluctuating course and tissue electivity. The lesions were not accompanied by profound dystrophic processes, and did not hinder the evolution of the chick embryo. In the first day, after the inoculation of bacterial suspension on to the chorioallantoic membrane, mild exudative inflammation was observed with soon disappearing hydropic and haemorrhagic phenomena, mainly focal in nature. The rapid evolution of the hydrops is due to the great hydrophilia of the embryonic tissue, but — because of its limited extent — the hydrops does not impair the functions of the embryo. The hyperhaemorrhagic phenomena are mild, not purpuric in appearance and cannot be used for the determination of the pathogenicity of strain 115 in the same manner as the pathogenecity of other micro-organisms e.g. *P-pestis* as established Budding and Womack (1941), Jawetz and Meyer (1944) and Knothe (1952). Sub-

sequently, the inflammatory reaction became productive in character: the mesodermic and hyperplastic processes appeared in the ectodermical layer as foci consisting of leucocyte infiltration and limited granuloma-like heaps of macrophages. The latter appeared at the site of introduction of the bacterium suspension; unlike virulent tubercle bacilli, they failed to disseminate. The macrophage heaps had no well-defined granulomatous structure, their cellular elements did not undergo profound dystrophic processes; in most cases macrocystic vacuolate dystrophia evolved. The hyperplastic processes were manifested in the ectodermal layer by string-like fibres and papillary increments 6—7 days after inoculation. The simple epithelium of the ectoderm became stratified, but keratinization of the superficial layers was never observed. Unlike the normal ectodermal epithelium, the proliferative epithelium consisted of spinous cells without the differentiated cell structures characteristic of the simple epithelium. The multiplying cells of the ectodermal layer formed string-like fibres and papillary increments from the compact cell complexes in the ectodermal layer. Unlike the proliferations brought about by substrain 1 of BCG, the epithelial proliferations due to strain 115 penetrated the mesodermal layer, but did not form filaments consisting of flat cells, nests and epidermoid "pearls", in contrast with the corresponding formations observable after introduction of virulent tubercle bacteria or some other antigens which are reminiscent of epidermoid cancer (Klod 1918; Stevensen 1918; Huxley and Marrey 1924; Campbell 1949; Rapoport 1959; Dikhno, Sorokina and Shimkevich 1963). Profound dystrophic lesions were not observed in the cells of epithelial proliferations, only less defined hydropic swelling and cystic vacuolization appeared. Mild hyperaemic infiltrative phenomena were observable in the subcutaneous connective tissue and in the viscera of the chick embryo. In spite of this, the excitation due to strain 115 neither slowed down nor disturbed the growth of the embryo.

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In the snake *Vipera ammodytes ammodytes*, after the injection of BCG only the well-defined pattern of the malpighian bodies were visible in the spleen. At the site of inoculation, caseous foci resembling those seen by Sorgo and Suess (1907), after injection of *M. tuberculosis typus humanus*, were not formed, Macroscopically, a dilatation of the germinative centres of the malpighian bodies 2—3 times the original size, and diffuse infiltration with pseudoeosinophilic leucocytes of the white and red pulp of the spleen were visible. Infiltration with pseudoeosinophiles was found in other organs,

too. In some cases catarrhal focal pneumonia without acid-fast bacteria was formed in some parts of the lungs.

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The above experiments have shown that, like BCG, the attenuated tubercle bacterium strain 115 gives rise to non-specific, hyperplastic-proliferative processes in the reticulohistiocyte system and limited proliferation of epitheloid cells at the site of injection, in the regional lymph-nodes and in the spleen. The exudative component of the inflammatory process appears to be more pronounced and develops more rapidly than after inoculation of BCG. A similar picture is observable after injection of the Brazilian BCG substrain No. 626 (Moro). The immunity achieved with the attenuated tubercle bacterium strain 115 in guinea-pigs is substantial, and it is somewhat stronger than that induced by BCG.

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CONCLUSIONS OF THE SCIENTIFIC CONFERENCE CONCERNING THE STRAIN W 115

The Microbiological Research Group of the Hungarian Academy of Sciences has organized a scientific meeting in order to discuss the results obtained in the study of the attenuated *M. tuberculosis* strain W 115 and to decide on the further tasks.

Participants of the meeting held on October 27 and 28, 1965, were: Prof. A. T. Kravtschenko, Director of Tarasievitch Institute of Vaccine Control (Moscow, USSR), Dr. T. B. Yablokova, Head of BCG and Tuberculin Laboratory, Tarasievitch Institute of Vaccine Control, (Moscow, USSR), Dr. L. Šula, Head of Department of Microbiology, Institute of Tuberculosis (Prague, Czeshoslovakia), Dr. D. Stoyanov, Head of Department of Microbiology, Ministry of Health (Sofia, Bulgaria), Dr. I. Földes, Scientific Director of the National Korányi Institute of Tuberculosis (Budapest, Hungary), Dr. L. Lugosi, Head of BCG Laboratory, State Institute of Hygiene (Budapest, Hungary), Dr. D. Mosolygó, Deputy Department Director, Ministry of Health (Budapest, Hungary) and Dr. L. Medve, Departmental Director of the Health Scientific Council (Budapest, Hungary), Prof. Gy. J. Weiszfeiler, Director of the Microbiological Research Group, Hungarian Academy of Sciences, Dr. V. Karassova, Chief of Laboratory of Microbiology, Microbiological Research Group (Budapest, Hungary).

Complete agreement was reached by the participants about the following items.

1. Examinations of the pathogenic properties of strain 115 have shown that the organism does not induce progressive tuberculotic changes in guinea-pigs and monkeys and, consequently, it meets the requirements for vaccine strains. As a result of histopathological findings on guinea-pigs, as compared with BCG, the residual virulence of strain 115 is equivalent (substrain Paris, dr. Földes) or somewhat higher (substrain Prague, Dr. Šula and substrain Moscow, Dr. Yablokova and Dr. Stoyanoff). It is very important that in monkeys infected with strain 115 (Weissfeiler) only bening symptoms developed which disappeared gradually.

2. The virulence of strain 115 remained unaltered on 7 continuous guinea-pig passages during 4 years. Therefore, the strain can be regarded as a stable attenuated culture. Thus, there is no danger that the strain may become virulent under certain unforeseen circumstances.

3. The immunogenicity of strain 115 was examined on guinea-pigs and monkeys. Guinea-pig experiments carried out by Dr. Jablokova revealed an immunogenicity identical with that of BCG; according to the guinea-pig experiments of Dr. Weiszfeiler and Dr. Stojanoff, immunogenicity of strain 115 was somewhat higher than that of BCG; however the difference was not significant. A substrain (115/9) obtained by selection gave more favourable results; therefore, further studies should be made with this strain. Immunization studies in monkeys (Weiszfeiler) yielded very favourable results, as strain 115 was shown to exert a considerably higher immunizing effect than BCG. Dr. Jablokova stressed that immunization experiments performed at the Institute of Microbiology, Tashkent, revealed the high immunogenecity of strain 115.

4. In view of the promising results, participants of the meeting agreed in the importance of carrying out further studies on strain 115, as this organism may be adequate for the production of a live vaccine at least equivalent with, or premusably superior to, the BCG vaccine. Dr. Sula thought that it is of interest to accomplish further studies in view of the elaboration of a vaccine which produces low degrees of tuberculin sensitivity.

5. As to the tasks and methods of further studies, the participants agreed on the following points. All investigators should employ identical methods so that the results should be comparable. As previously comparison with BCG should be included in all experiments. Large numbers of lyophilized ampoules of substrain 115/9 and of BCG substrain Paris will be deposited by Dr. Lugosi in the State Institute of Hygiene. Both strains will be given code numbers known only by Dr. Medve. Thus, neither of the investigators will know which of the code numbers represent strain BCG and strain 115. Superinfection will be made with strain H37Rv provided by the Microbiology Research Group. No pure animal strains are required for guinea-pig experiments; mouse experiments should primarily be carried out by use of pure strain Balb, although other mouse strains may also be employed. Guinea-pigs should be free from other infectious diseases and coccidiosis. Immunogenicity should be estimated according to the survival period of the immunized animals by using statistical methods recommended by W.H.O.

As non-compulsory examinations, studies on the following problems are advisable: intracellular multiplication and cytopathogenic effect of strain

115 on human diploid cells or animal monocytes; strain-specificity of tuberculin produced from strain 115; immune chemistry and antigenic structure of strain 115.

Experiments planed by Dr. Lugosi are satisfactory. However, it should be checked whether immunization experiments on groups, each containing 20 animals, are sufficient for statistical evaluation.

6. As to human experiments with strain 115, the following point of view was adopted. The above-mentioned experiments will be completed within a period of one year. If these show favourable results, it will be advisable to perform human experiments. The effect of strain 115 should be observed on both tuberculin positive and tuberculin negative individuals. The experiments should be performed on volunteers and on children with the approval of their parents.

7. The participants request the Director of the Tarasievitch Institute to organize a similar meeting in Moscow with the participation of the Tashkent Research Group at the end of 1966 or at the latest by April, 1967.

8. The participants desire that lectures held at the meeting should be published (preferably in English) by the Microbiology Research Group.

9. The participants of the meeting uniformly regard the discussions as useful and important for promoting a more rapid and reliable research programme.

CULTIVATION OF UNICELLULAR ALGAE IN ASSOCIATION WITH BACTERIA

by

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I. Continuous cultivation of Chlorella pyrenoidosa in sewage water

THE CULTIVATION of unicellular algae in association with bacteria contributes to the solution of important theoretical and practical problems. Heterotrophic bacteria decompose organic materials and produce carbon dioxide and meanwhile utilize oxygen which is excreted by algae. The latter organisms which are mainly autotrophs use the metabolic products and carbon dioxide formed by bacteria and gain energy for biosynthesis mainly from light. Thus, the circulation of organic and inorganic material is very suitably accomplished. Cultivation of algae is desirable for practical reasons as these organisms comprise an economic protein source for animal as well as human nutrition. In addition algae may play an important, possibly a guidable, role in biological sewage purification.

Caldwell (1946) and Myers (1948) examined mass culturing of algae in so-called oxidation basins. During extensive work, Oswald, Gotaas, Golueke and Kellen established pilot plants in Richmond (California) and Phoenix (Arizona). As a result of a 3-year observation period they recommended this method of sewage treatment. Meffert (1957) cultured a *Scenedesmus obliquus* algal strain in sewage with good results. Winberg (1964) in Minsk (USSR) found Scenedesmus and Chlorella strains suitable both in laboratory and in sewage plants. Nakamura (1961, 1964) examined mass cultivation of Chlorella for producing fodder both in artificial medium and in liquid manure. Krauss (1962), Mayer et al. (1964), Brown et al. (1964) studied the possibility of utilisation of wastes for mass cultures of algae with bacteria. Hua Zhu Tchena and Li Bin Dao have given account of extensive mass cultivation in liquid manure of *Scenedesmus obliquus* and *Chlorella pyrenoidea* which has been carried out in China since 1958. This method yielded good result in agricultural stock breeding.

These experiments provided no data as to the multiplication and taxonomic position of characteristic bacteria taking part in combined cultivation. Cooper (1962) showed that after the multiplication of algae the number of

enterococci and coliform bacteria decreases about 10—100 times. Wladimirova and Bazaitova (1961) and Maximova and Fedenko (1965) examined under experimental conditions the characteristics of combined cultivation

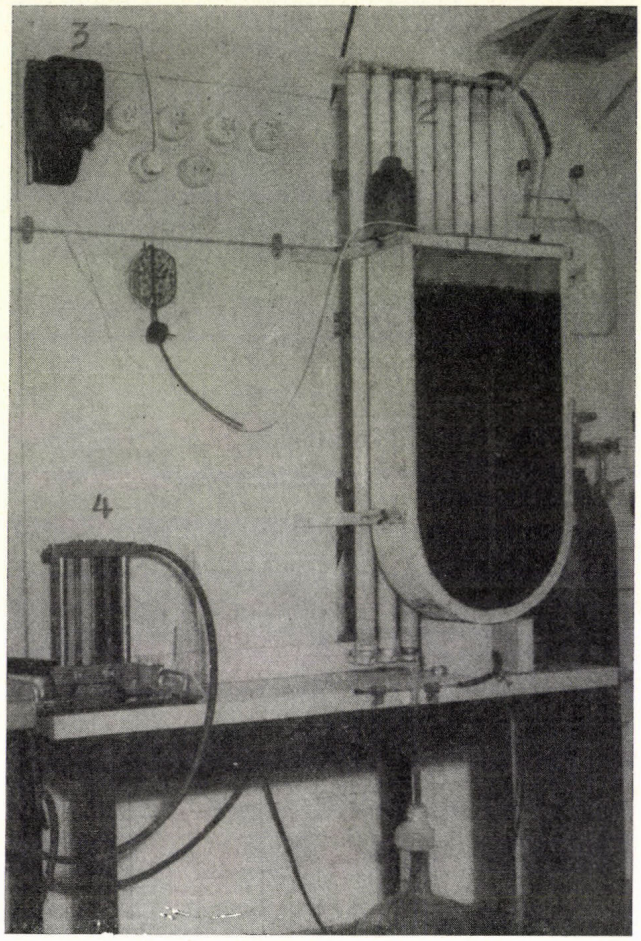

FIG. 1. Equipment for laboratory mass production of algae. 1 = cultivation container; 2 = illumination tubes; 3 = automatic connector; 4 = gas mixer and measuring rotameter

of Pseudomonads and *Bacillus cereus* and of Chlorella. The latter authors pointed out the unfavourable effect of the decrease in redox potential.

In our studies we wanted to examine the conditions for mass cultivation of algae in home sewage. Therefore it seemed necessary to isolate a suitable algal strain, to elaborate an adequate equipment and to examine the bac-

terial flora yielding favourable culturing conditions. A sedimented sewage originating from an activated sludge plant situated in the village Budakeszi was used. The characteristic properties of this sewage were extensively studied by Tóth, Gregács and Muhics (1962), who found that it contained 0.1—1.0 mg dissolved oxygen per litre. Our *Chlorella pyrenoidosa* MK1 strain was isolated from this sewage after a 14-day incubation in day light. The culture was identified by Dr. G. Szemes of the Danube Water Research Station of the Hungarian Academy of Sciences. An impure culture containing 10^8 cells per ml in Enebo-Johnson medium was used as an inoculum.

In the experiments a 25 litre container with an effective illuminated surface of 0.25 m^2 was used (Fig. 1). The transilluminated layer was 10 cm thick; the semicircular formation of its lower part and internal deflecting plates were necessary to promote good circulation conditions and to avoid dead spaces. Stirring of the algal suspension was performed by bubbling air containing 2% carbon dioxide through the medium at a rate of 4 litres per minute. A 40 watt Tungsram F7 fluorescent lamp yielding a 5500 lux intensity served as a light source. Illuminated periods lasted for 16 hours and dark periods for 8 hours. At the beginning of cultivation the algal count was 4 million per ml; after some days of culturing the number of algae reached 25—50 million cells per ml. From that time onward a daily amount of 4—6 litre suspension was removed and the volume of the medium was brought up with sewage. Algae were centrifuged, or later, as recommended by Chinese authors, precipitated by adding 6% of saturated calcium hydroxide solution. Organic material content of sewage and alga-free filtrate of cultures were estimated from oxygen consumption determination with the potassium permanganate method of Kubel and Thiemann. Bacteriological analysis involved the estimation of total counts at 22 and 37 °C, coli titre and classification of the predominating bacteria into genera. The experiments were performed between May and December, 1965.

Results

In the summer months the experiments had to be discontinued after 10—14 days because of the appearance of filamentous algae. Three experiments performed during the summer and autumn months are summarized in Table I. In an experiment which began November 11 and lasted for 50 days, 112 litres of suspension yielding 118.8 g dry weight algae was harvested. In spite of the fact that fresh sewage was regularly added to the culture, the organic material content of the suspension decreased by about 50% due primarily to bacterial activity. Algal counts corresponded in the first 3 experiments to 69—91

TABLE I

Experiment		Total algae yields		Average algae cells		Average oxygen consumption (mg/l)		Total bacterial counts 37 °C (average)	
No.	Duration (days)	(litre)	(g)	g/l	10^6/ml	Sewage	Culture fluid	Sewage 10^3/ml	Algae Culture 10^3/ml
I.	18	36	24.6	0.68	61.0	37.5	25.1	773	617
II.	14	35	26.75	0.89	70.5	34.8	24.9	604	604
III.	11	38.7	34.15	0.88	71.0	36.8	25.7	772	703

TABLE II

Experiment		Total algae yields		Average algae cells		Average bacterial and E. coli counts 10^6/ml					
						Sewage			Algae culture		
No.	Duration (days)	(litre)	(g)	g/l	10^6/ml	20 °C	37 °C	Coli	20 °C	37 °C	Coli
IV	50	112.0	118.7	1.06	25.77	1021	310	75.1	129	109.1	2.77

million per ml, while in the fourth (Table II) experiment to approximately 25 million per ml. The smaller counts observed in the fourth experiment were presumably due to a clumping of algae. Bacteriological examinations revealed that the total count decreased only slightly; in one experiment, however, extremely low counts were observed (3—80 times lower than the usual). With the exception of two cases the coli count considerably decreased (to 0.1—0.01). While in sewage a wide variety of organisms belonging to the family *Enterobacteriaceae* were encountered, the algal culture contained only 3—5 kinds of bacteria. Mainly *E. alkaligenes*, *Neisseriae* and *Micrococci* were isolated. Thus it may be concluded that a relatively stable biocoenosis developed between algae and bacteria which was favourable for the multiplication of both.

Summary

Chlorella pyrenoidosa strain MK1 isolated from sewage multiplied intensively in association with some bacteria as *E. alkaligenes*, *Neisseria and Micrococcus* in home sewage water.

As the organic material content and coli titre of sewage decreased, the multiplication of algae improved the biological purification of sewage.

Establishment of a pilot plant for the production of fodder protein by continuous cultivation of algae in the presence of bacteria seems reasonable.

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II. Continuous cultivation of thermophilic Chlorella pyrenoidosa strain in synthetic medium

As to optimal temperature of multiplication, unicellular algae, as Chlorella and Scenedesmus are mesophilic, i.e. they grow between 20—30 °C. Sorokin and Myers (1953) isolated a thermophilic Chlorella (strain Tx 71105) which multiplied intensively at 39 °C. Further investigations yielded more thermophilic algal strains. In Japan Tamiya (1956), in the USSR Rubenchik, Kordjum and Lazurkevich (1961) adapted a *Chlorella vulgaris* strain to grow at 39—41 °C. Biosynthetic processes in thermophilic algae are more intensive and, therefore, they are used in Japan, for mass cultivation especially at higher summer temperatures. Recently Blasco (1965), Vela and Guerra (1966) studied the dynamics of multiplication of the thermophilic Chl. pyrenoidosa strain TX 71105 in mixed cultures with some bacteria. In Hungary the elaboration of mass cultivation of thermophilic algae would enable us to ensure optimal conditions for growth throughout the whole year by use of thermal waters.

Our *Chlorella pyrenoidosa* (strain T91) which multiplies intensively at 37 °C was isolated from the water of a thermal spring in Debrecen. Culturing conditions for this strain were examined in Enebo—Johnson medium at different temperatures and illuminations. The bacterial flora of the culture was also examined as this algal strain always multiplied in the presence of bacteria.

Equipment

Chlorella strain 9192 was cultured at 24 °C room temperature in Erlenmeyer flasks placed close to the window on a glass plate illuminated from below with three 40 W Tungsram F7 fluorescent lamps yielding a total of 3000 lux intensity of light. The suspension was stirred with a motor and supplied with air containing 2% carbon dioxide. Cultivation at 37 °C was

performed in a thermostat illuminated with two 200 W F7 fluorescent lamps, yielding 1000 lux intensity. In the latter experiments no stirring motor was used.

Results

Intensity of multiplication at room and thermostat temperatures cannot be compared, as the light intensity, due to an exposure to daylight and to a more intensive artificial light, was much higher for room than for thermostat temperature cultures.

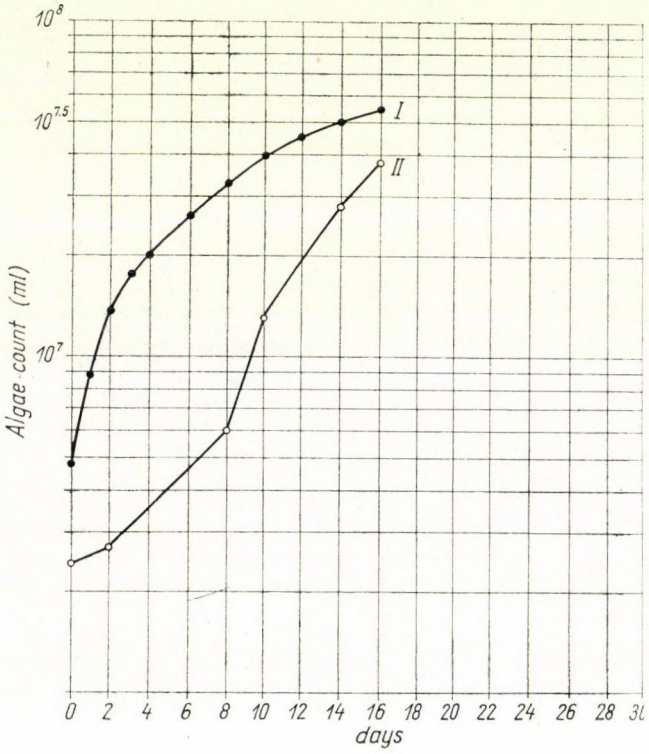

FIG. 1. Cultivation of thermophilic *Chlorella pyrenoidosa* T91 strain at 37 °C in two experiments

Multiplication at 24 °C was so intensive that fresh medium could be substituted for about 50% of the culture liquor at 2—3 day intervals. The algal counts increased 3—4 times during these intervals and reached 75—115 million cells per ml.

Multiplication at 37 °C was also intensive; the counts increased 5—6 times during 5 days and reached 40—100 million cells per ml. The results of two experiments are shown in Fig. 1. This strain grows well also at 39 °C.

Bacteriological analysis showed that room and thermostat temperature cultivation yielded about the same number of bacteria (100,000—400,000 total counts). An *E. alkaligenes* and a *Micrococcus* strain predominated in the cultures. Since the synthetic medium contained urea as the only organic substance, it may be assumed that the multiplication of these microorganisms was facilitad in symbiosis with Chlorella.

Accordingly, *Chlorella pyrenoidosa* strain T91, in addition that it grows well at 37 °C, is capable of intensive multiplication in the presence of urea as sole organic material. This property makes possible its employment for mass cultivation under semiplant conditions by the use of thermal springs.

Summary

A thermophilic *Chlorella pyrenoidosa* strain multiplying well at 37 °C was isolated from the water of a hot spring.

Chlorella pyrenoidosa strain T91 proved suitable for mass cultivation with *E. alkaligenes* and *Micrococcus* at 37 °C.

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